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(54) Title: COMPOSITIONS AND METHODS FOR MODULATING SERUM CHOLESTEROL

(57) Abstract

Compositions and methods are provided for modulating serum cholesterol in a subject mammal. In one aspect, the invention features novel anti–lipemic drugs that include at least one identified effector of the Low Density Liproprotein (LDL) receptor and at least one identified serum cholesterol inhibitor. In a particular aspect, the drugs include one identified sphingolipid or protein modifying same linked to one identified serum cholesterol inhibitor. Additionally provided are methods for identifying anti–lipemic drugs capable of modulating the LDL receptor and specifically SREBP–1 maturation, including assays designed to identify pharmacoligical drugs capable of stabilizing or reducing serum cholesterol levels in a mammal and particularly a human patient.

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COMPOSITIONS AND METHODS FOR MODULATING SERUM CHOLESTEROL

This application claims the benefit of U.S. provisional application number 60/121,447, filed February 24, 1999, which is incorporated herein by reference in its entirety.

STATEMENT OF GOVERNMENT INTEREST

Funding for the present invention was provided in part by the Government of the United States by virtue of National Institute of Health Grants R0-1 DK-31722 and P50-HL4812. Thus, the Government of the United States has certain rights in and to the invention claimed herein.

FIELD OF THE INVENTION

The present invention relates to compositions and methods for modulating serum cholesterol. In one aspect, the invention features novel anti-lipemic drugs that include at least one identified effector of the Low Density Lipoprotein (LDL) receptor and at least one identified serum cholesterol inhibitor. In a particular aspect, the anti-lipemic drug includes at a sphingolipid or protein modifying same linked to the serum cholesterol inhibitor. Additionally provided are methods for using the anti-lipemic drugs to significantly stabilize or reduce serum cholesterol levels in a subject mammal and particularly a human patient.

BACKGROUND OF THE INVENTION

There is nearly universal agreement that cholesterol is a key lipid constituent of cell membranes. Cholesterol is generally understood to be essential for normal growth and viability of most higher organisms. Too much serum cholesterol has been correlated with life threatening lipid related diseases including hyperlipoproteinemia,

stoke, coronary heart disease, and especially artherosclerosis and related conditions. See generally Stryer, L. (1988) in *Biochemistry*, 3rd Ed. W.H. Freeman and Co. New York, pp. 547-574; and Brown, M.S. and Goldstein, J.L. (1993) in *The Pharmacological Basis of Therapuetics* (8th Ed.) Gilman, A.G. et al. eds. McGraw-Hill/New York, pp. 874-896.

The regulation of serum cholesterol in mammals and particularly primates has attracted significant attention. It is often reported that regulation of cholesterol homeostasis in humans and other mammals involves regulation of cholesterol production, bile acid biosynthesis and catabolism of specific serum cholesterol carriers. Important serum cholesterol carriers are called LDL (low density lipoprotein) particles. The LDL receptor has been reported to facilitate internalization of the LDL particle into those cells in need of cholesterol. See e.g., Brown, M.S. and Goldstein, J.L. (1986) *Science* 232: 34-47; and Goldstein, J.L. and Brown, (1986) *Nature*, 348: 425; and references cited therein.

The LDL receptor has been disclosed as impacting serum cholesterol levels in humans. For example, there has been recognition that cells with enough cholesterol do not make sufficient LDL receptors, thereby reducing or even blocking uptake of cholesterol by the cell. In this instance, serum cholesterol levels rise substantially which can contribute to the development or severity of disease. Conversely, cells in need of cholesterol often have capacity to make more LDL receptors, thereby facilitating a decrease in serum cholesterol. Accordingly, there has been specific attention focused on regulating the LDL receptor as one therapeutic approach for stabilizing or reducing serum cholesterol levels in human patients.

In particular, it has been reported that transcription of the LDL receptor gene is suppressed when sterols accumulate and induced when sterols are depleted. Sterol sensitivity is thought to be conferred by a 10 basepair (bp) sequence upstream of the LDLr gene known as the sterol regulatory element (SRE). It has been disclosed that

the mature form of the sterol regulatory element binding protein-1 (SREBP-1) binds to the SRE and promotes transcription.

There have been additional reports that the activity of SREBP-1 is influenced by sterol induced proteolysis. There is recognition that the SREBP-1 proteolysis is impacted in some settings by a cell receptor termed "cytokine tumor necrosis factor" (TNF-α).

In particular, the TNF-α receptor has been reported to influence a wide range of biological effects. However, the TNF-α receptor remains incompletely characterized. Elucidation of TNF-α pathways is sometimes complicated by presence of at least two TNF receptors. The receptors share some common downstream effectors but also signal via receptor specific pathways. See the references cited below for additional disclosure relating to the TNF-α receptor.

There has been understanding that one consequence of TNF signaling is the activation of neutral sphingomyelinase (N-SMase). Neutral sphingomyelinase is a membrane bound enzyme that catalyzes the hydrolysis of sphingomyelin to ceramide and phosphocholine at a pH optima of 7.4. The role of neutral sphingomyelinase in signal transduction has primarily been related to ability to generate the lipid second messenger ceramide. In addition to TNF-α, Fas receptor ligand, vitamin D_3, interleukin-1β, nerve growth factor, anti-CD28 antibodies and γ-interferon have all been shown to increase ceramide levels.

In particular, sphingomyelinases type-C (E.C. 3.1.4.12) are a group of phospholipases that catalyze the hydrolytic cleavage of sphingomyelin via the following reaction (1).

Sphingomyelin ----> Ceramide + Phosphocholine (1)

See S. Chatterjee, *Adv. Lipid Res.*, 26:25-48 (1993); S. Chatterjee et al., *J. Biol. Chem.*, 264:12,534-12,561 (1989); and S. Chatterjee et al., *Methods in Enzymology, Phospholipase*, 197:540-547 (1991).

In addition to the biological roles of sphingomyelin and ceramide in signal transduction pathways involving cell regulation, more recent evidence has emerged suggesting that sphingomyelinases may be involved in cholesterol homeostasis and particularly induction of LDL receptor activity. See S. Chatterjee, *Advances in Lipid Research*, 26:25-48 (1993). Additional work supports a possible role of ceramide in programmed cell death and/or "apoptosis" and activation of the gene for nuclear factor (NF)-kB. See A. Alessenko and S. Chatterjee, *Mol. Cell. Biochem.*, 143:169 (1995).

It has been suggested that certain enzymes involved in making cholesterol exert a significant effect on cholesterol homeostasis. Accordingly, there has been substantial interest in identifying drugs with capacity to modulate these enzymes especially to stabilize or reduce serum cholesterol to tolerable ranges. Illustrative agents include commercially available serum cholesterol inhibitors such as fluvastatin, simvastatin, lovastatin, pravastatin, and atorvastatin. See Brown, M.S. and Goldstein, J.L. (1993), *supra* for additional disclosure relating to these and other agents such as mevinolin (compactin).

Although some clinical benefit has been reported to follow use of these and other serum lowering agents, there have been reports of significant side-effects. See e.g., Brown, M.S. and Goldstein, J.L. (1993), *supra*; and *Physicians' Desk Reference* 1997 (51st ed.) Medical Economics Co. Accordingly, there is a need to have drugs that exhibit more desirable characteristics such as enhanced potency and better patient tolerance. There is a specific need to reduce levels of administered cholesterol lowering agents for some patients.

There is also a need to identify drugs that can modulate the SREBP-1 protein and especially the LDL receptor. Moreover, methods for identifying pharmacological drugs of interest by automated, high throughput drug screening have become increasing relied upon in a variety of pharmaceutical and biotechnology drug development programs. Unfortunately, requisite drugs for such high throughput screening assays are not widespread. A significant reason for lack of progress in this area is insufficient understanding of molecules (i.e. effectors) that impact SREP-1 and the LDL receptor.

It thus would be desirable to have anti-lipemic drugs with dual capacity to modulate the LDL receptor and serum cholesterol levels. It would be particularly desirable if such anti-lipemic drugs could be administered to subject mammal at doses near or below those presently used with many serum cholesterol inhibitors. It would be further desirable to have effective in vitro and in vivo assays for identifying drugs with potential to modulate the LDL receptor particularly involving SREP-1 protein maturation.

SUMMARY OF THE INVENTION

The present invention generally relates to compositions and methods for modulating serum cholesterol in a subject mammal. In one aspect, the invention features novel anti-lipemic drugs that include at least one identified effector of the Low Density Lipoprotein (LDL) receptor and at least one identified serum cholesterol inhibitor. In a particular aspect, the drugs include one identified sphingolipid or protein modifying same linked to one identified serum cholesterol inhibitor. Additionally provided are methods for identifying anti-lipemic drugs capable of modulating the LDL receptor and specifically SREBP-1 maturation, including assays designed to identify pharmacological drugs capable of stabilizing or reducing serum cholesterol levels in a mammal and particularly a human patient.

We have discovered a wide spectrum of compositions and methods for treating or preventing disorders modulated by cholesterol. Sometimes the disorders will be referred to herein as "cholesterol related disorders" or a similar term. More specifically, we have identified anti-lipemic drugs that include at least one identified effector of the LDL receptor, and particularly an effector of SREBP-1 and at least one identified serum cholesterol inhibitor. Particular anti-lipemic drugs of this invention usually have one of each component although drugs having multiple effectors and inhibitors (e.g., between from about 2 to 5 of each) are contemplated. Preferred anti-lipemic drugs feature specifically defined characteristics such as capacity to stabilize or reduce serum cholesterol levels in a subject mammal as determined by in vitro or in vivo assays described below.

More specifically, the present invention provides a variety of specific anti-lipemic drugs and methods for using same for the treatment or prevention of one or more than one cholesterol related disorder in a subject mammal. Illustrative disorders are known in the field and include hyperlipoproteinemia including hypercholesterolemia, stroke, obesity, compulsive eating disorders, cardiac disease including atherosclerosis, cerebral atherosclerosis, cholesteryl ester storage disorder, liver disease including organ transplantation failure and cirrhosis; diseases of the biliary system, and viral infection, particularly those infections facilitating encephalitis or related disorders.

Particular anti-lipemic drugs in accord with this invention include one SREBP-1 effector and one synthetic or semi-synthetic inhibitor of an enzyme associated with cholesterol biosynthesis. Preferred enzymes have been extensively characterized and include 3-hydroxy-3-methylglutaryl (HMG) CoA reductase and HMG CoA synthetase. Additionally contemplated anti-lipemic drugs feature, as the effector component, an identified caspase, particularly the cpp32 protease (caspase-3), neutral sphingomyelinase (N-SMase), ceramide, SREBP-1 (precursor), or SREBP-1 (mature). Effective fragments of the N-SMase, cpp32 protease, SREBP-1 (precursor),

or the SREBP-1 (mature) protein are contemplated as effector molecules within the scope of this invention.

Additionally specific anti-lipemic drugs include one effector of SREBP-1 which effector can be a sphingolipid, e.g., sphingmyelin or ceramide; or N-SMase or an effective fragment thereof. In embodiments in which the anti-lipemic drug includes ceramide, that ceramide molecule is preferably naturally-occurring (ie. can be isolated in substantially pure form from a biological source). A more preferred ceramide for use in the drug is any one of C-2, C-4, C-6 or C-8 ceramide. A preferred N-SMase molecule is encoded by specific nucleotide sequences disclosed herein including those encoding enzymatically active forms of that enzyme and effective fragments thereof. Preferred effectors in accord with this invention demonstrate substantial capacity to modulate the LDL receptor and especially maturation of the SREBP-1 protein as determined by specific assays described below.

As discussed, particular anti-lipemic drugs of this invention include a suitable SREBP-1 effector such as sphingolipid, particularly a sphingomyelin or ceramide, N-SMase or effective fragment thereof, although other drugs may include other effectors as needed. In this embodiment, the anti-lipemic drug further includes the inhibitor of HMG CoA reductase. It is generally preferred that the effector and the inhibitor are be combined in a way to facilitate function for which the drug was intended. A preferred function is to stabilize or reduce serum cholesterol as determined by a conventional in vivo assays defined below. In most instances, covalent attachment between the effector and the inhibitor will be preferred although other associations will be suitable for some applications. Preferred cholesterol inhibitors have recognized capacity to inhibit the reductase, thereby lowering serum cholesterol. Illustrative inhibitors include commercially available serum cholesterol inhibitors acceptable for human use, e.g., fluvastatin, simvastatin, lovastatin, pravastatin, mevinolin (compactin), atorvastatin; or a clinically acceptable derivative thereof.

In a particular embodiment, the anti-lipemic drugs include one effector of the SREBP-1 protein, e.g., the N-SMase or effective fragment; or a sphingolipid. In this example, the effector is also preferably associated with the inhibitor of HMG CoA reductase. By the term "associated" or related term is meant that the SREBP-1 effector and the inhibitor are attached by at least one bond preferably at least on covalent bond. Particular examples of bonding are described below. In some instances, the association can also be provided by a suitable combination of covalent and non-covalent chemical bonds. Alternatively, association between the SREBP-1 effector and the inhibitor can be provided by essential co-administration of the effector and the inhibitor to a desired subject mammal. More specific methods for making and using the drugs of this invention are provided in the discussion and examples which follow.

In one embodiment, the anti-lipemic drug includes the sphingolipid attached to the inhibitor by at least one covalent bond. As noted, preferred are recognized cholesterol inhibitors such as fluvastatin, simvastatin, lovastatin, pravastatin, mevinolin (compactin), atorvastatin. In this illustration, the sphingolipid is preferably ceramide or a related molecule, particularly any one of the preferred ceramides described previously, which ceramide is covalently linked to a reactive hydroxyl group on the inhibitor molecule. Also in this example, the hydroxyl group of the inhibitor is usually covalently linked to a reactive carbon atom on the ceramide such as the C-3 carbon.

Additional anti-lipemic drugs of this invention include at least one bifunctional spacer group, typically a heterobifunctional spacer group, which group spaces the SREBP-1 effector from the inhibitor or other drug moiety. A particular example of this type of anti-lipemic drug includes one SREBP-1 effector covalently linked to one heterobifunctional spacer group. That spacer group is preferably covalently linked to the serum cholesterol inhibitor. Typically, the bifunctional spacer

is linked to suitably reactive chemical group on the effector and the inhibitor, usually specifically reactive carbon atoms and hydroxyl groups, respectively.

Further anti-lipemic drugs in accord with the present invention include one effector of SREBP-1 such as the neutral sphingomyelinase (N-SMase) or an effective fragment thereof. A preferred drug includes the N-SMase or the fragment in association with an inhibitor of HMG CoA reductase or HMG CoA synthetase as described previously. Preferred examples of the N-SMase and fragment are provided in the examples and discussion which follow.

Further contemplated anti-lipemic drugs include the effector of SREBP-1, preferably the neutral sphingomyelinase (N-SMase) or the fragment thereof; which effector is covalently linked to one inhibitor of the HMG CoA reductase. Preferred inhibitors of the reductase have already been discussed. Preferably, the covalent linkage is made by binding a chemically reactive group on the enzyme or fragment, preferably an amide bond. More particular anti-lipemic drugs are disclosed below featuring an amide linkage between the enzyme or fragment and the serum cholesterol inhibitor.

Preferred anti-lipemic drugs of this invention are generally formulated to suit intended use and specifically include those drugs formatted for topical or related use. Additionally, the invention includes anti-lipemic drugs that include components sufficient to provide the drug as a liposome formulation suitable for in vitro or in vivo use. Methods for making and using such preferred drugs are described below.

In general, therapeutic methods in accord with this invention include administering to a subject, particularly a mammal such as a primate, especially a human, a therapeutically effective amount of at least one anti-lipemic drug of interest. That drug can be administered as a sole active agent. Alternatively, the anti-lipemic drug can be administered in combination with other drugs or agents exhibiting a

desired pharmacological activity. In most cases, the amount of anti-lipemic drug use will be one which exhibits good activity in a standard in vitro or in vivo assay described below.

As discussed, the anti-lipemic drugs of this invention advantageously provide dual "anti-cholesterol" activity, ie, by increasing LDL receptor activity, particularly by enhancing LDL receptor levels; and by reducing serum cholesterol levels. Particular in vitro and in vivo assays to detect and quantitate these activities are provided below and in the discussion and examples which follow.

As an illustration, preferred anti-lipemic drugs of this invention are capable of stimulating production of the mature form of SREBP-1 (maturation) by at least about 2 fold, as determined by a standard SREBP-1 proteolysis (maturation) assay. That assay is provided below and generally involves monitoring in a time and dose dependent manner, the maturation of the SREBP-1 protein. Mature SREBP-1 protein is believed to move to the nucleus and stimulate production of LDL receptor.

Additionally preferred anti-lipemic drugs of this invention are capable of increasing LDL receptor mRNA levels by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80, or 90% as determined by Northern blot or related mRNA detection assay. An exemplary Northern blot assay for detecting and optionally quantitating LDL receptor mRNA levels are provided below.

Also preferred anti-lipemic drugs of this invention exhibit an ID_{50} of between from about 20%, 30%, 40%, 50%, 60%, or 70% to about 90% as determined in a standard HMG CoA reductase assay. In this assay, the activity of the reductase enzyme is monitored in the presence and absence (control) of the anti-lipemic agent. An example of the standard HMG CoA reductase assay is provided below.

Further preferred anti-lipemic drugs are capable of significantly reducing serum cholesterol as determined by a standard serum cholesterol assay. Preferably, an administered anti-lipemic drug is capable of reducing serum cholesterol in a subject mammal by at least about 5%, 10% to 20% or 30%, 40%, 50%, 60% or 70%. An example of the assay is described below. Typically, the reduction in serum cholesterol is monitored with respect to a suitable control subject. The serum cholesterol assays are optimally performed in vivo and preferably include use of a recognized animal model such as specific rabbit and mouse strains provided below.

Preferred animal models for use in the serum cholesterol assay or other suitable assay disclosed herein are generally recognized test systems for an identified cholesterol related disease. Typically such animal models include commercially available in-bred strains of rabbits or mice, e.g., the Watanabe heritable hyperlipidemic rabbit and the apolipoprotein E negative mouse. In this example, the reduction in serum cholesterol can be evaluated using well-known testing strategies adopted for use with the specific animal model. However for some applications it may be useful to test a desired anti-lipemic drug on a normal ("wild-type") animal such as those genetically defined (e.g., isogenic) wild-type animal strains known in the field.

The anti-lipemic drugs of this invention are preferably tested by at least one and preferably all of the standard assays summarized above. Preferred are anti-lipemic drugs that demonstrate about the stated activity ranges in one or more of the assays.

Significantly, use of multiple testing strategies (e.g., a combination of one in vitro and/or in vivo assays) with a single anti-lipemic drug can extend the selectivity and effectiveness of the testing as needed. That is, the testing strategy can be tailored for treatment or prevention of a particular cholesterol related disease or group of patients if required.

Such broad spectrum testing provides additional advantages. For example, preferred anti-lipemic drugs have capacity to enhance LDL receptor activity (typically by enhancing production of the LDL receptor) and provide for a reduction in serum cholesterol level. Thus by providing such dual "anti-cholesterol" activity, the invention is a significant advance over prior therapies and agents that have been reported to reduce serum cholesterol in one way, usually by targeting cholesterol biosynthesis. Accordingly, preferred anti-lipemic drugs of this invention feature better activity, can be administered at lower dosages then prior agents. Patient tolerance of the anti-lipemic drugs will also be positively impacted.

In another aspect, the invention includes methods for modulating and particularly reducing serum cholesterol level in a mammal. In this embodiment, the methods generally include administering to the mammal a therapeutically effective amount of at least one and typically one of the anti-lipemic drugs disclosed herein.

Also provided are methods for modulating LDL receptor levels in a mammal in which the method includes administering to the mammal a therapeutically effective amount of at least one and typically one of the anti-lipemic drugs disclosed herein.

The present invention also provides methods treating a disorder in a mammal having or suspected of having high serum cholesterol levels. In this embodiment, the method includes administering to the mammal a therapeutically effective amount of at least one of the anti-lipemic drugs disclosed herein. A preferred mammal is a primate and especially a human patient, e.g, those susceptible to coronary heart disease, obesity, eating disorders or other cholesterol related disorders described herein. Accordingly, the methods are especially applicable to a subject mammal such as a human patient who has been diagnosed as having, is suspected of having, or is susceptible to a high serum cholesterol level, e.g., through adverse genetic or dietary influences.

Also provided by this invention are methods for modulating serum cholesterol level in a mammal in which the method includes administering to the mammal a therapeutically effective amount of at least one of the anti-lipemic drugs disclosed herein. In this embodiment, the SREBP-1 effector is preferably neutral sphingomyelinase (N-SMmase) or an effective fragment thereof; or a sphingolipid such as ceramide. Preferred methods employ a primate such as a human patient. Preferred anti-lipemic agents for use in the methods are typically tested for activity using a recognized animal model for a cholesterol related disorder and especially atherosclerosis, e.g., the Watanabe heritable hyperlipidemic rabbit or an apolipoprotein E negative mouse discussed previously.

Additionally contemplated are methods for modulating LDL receptor in a mammal in which the methods include administering to the mammal a therapeutically effective amount of at least one of the anti-lipemic drugs disclosed herein. The modulation is preferably an increase in the synthesis (or sometimes decrease in the degradation of) the LDL receptor. In this example, the SREBP-1 effector is neutral sphingomyelinase (N-SMase) or an effective fragment thereof; or a sphingolipid such as ceramide. Methods for evaluating an increase or decrease in LDL receptor levels are known in the field and involve, e.g., molecular and immunological approaches using anti-LDL antibodies capable of detecting and quantitating LDL receptor in vitro or in vivo.

Particular methods of this invention involve use of at least one suitable anti-lipemic drug which includes one effector of SREBP-1 associated with an identified inhibitor of serum cholesterol as discussed herein. In this example, that effector is preferably a sphingolipid such as ceramide. Preferred examples of ceramide include naturally occurring ceramide and other ceramide forms as discussed previously. As discussed, preferred methods are conducted using a mammalian subject such as a primate and especially a human patient who has been diagnosed as having, is suspected of having, or is susceptible to a cholesterol related disorder as disclosed.

In an embodiment of the methods disclosed herein, the anti-lipemic drug is preferably disposed as a liposome formulation. In this example, the liposome formation can be compatible for hepatic administration in accordance with standard practice. Also in this example, the liposome formulation can be administered to the liver or associated organ in a human patient according to standard medical techniques involving, e.g., oral, intramuscular, intraperitoneal, administration via a stent or related implementation. Particular routes of administration are provided below.

Other aspects of the invention are discussed below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is graph showing effect of TNF-α on neutral sphingomyelinase (N-SMase) activity.

Figures 2A and 2B are graphs illustrating effects of TNF-α, sphinogmyelinase, and C_2 -ceramide on the kinetics of SREBP-1 maturation. 2A) kinetics of SREBP-1 maturation, 2B) ratio of immature/mature SREBP-1 versus time.

Figure 2C is a representation of a Western immunoblot showing expression of TNF-α, sphingomyelinase and C_2 ceramide.

Figures 3A-C are graphs showing effects of TNF-α (3A) , sphinogmyelinase (3B) , and C_2 -ceramide (3C) on SREBP-1 maturation.

Figure 4 is a representation of a Western immunoblot showing effect of anti-N-SMase antibodies on TNF-α-induced SREBP-1 maturation.

Figures 5A-D are representations of indirect immunofluorescence micrographs showing SREBP-1 expression in cells.

Figures 6A-6D are representations of gels showing results of electrophoretic mobility shift assays.

Figure 7 is a model showing how TNF-α induces SREBP-1 proteolysis(maturation) and mobilizes membrane cholesterol in human hepatocytes. Effectors of the LDL receptor and particularly SREBP-1 are shown schematically.

Figure 8 is a representation of a Western immunoblot showing N-SMase protein in cells expressing increasing amounts of a recombinant vector encoding the N-SMase (PHH1 lanes 3-6; PHH11 lane 9).

Figure 9 is a representation of a Northern blot showing expression of the vectors encoding the N-SMase protein (lane 2 PHH1; lane 3 PHH11).

Figure 10 is a representation of a Western immunoblot illustrating SREBP-1 expression and maturation in cells.

Figure 11 is a drawing showing a nucleotide sequence (SEQ ID NO:1) of isolated cDNA encoding human N-SMase.

Figure 12 is a drawing illustrating the deduced amino acid sequence (SEQ ID NO:2) of human N-SMase.

Figure 13 is a drawing showing examples of particular anti-lipemic drugs, target organs and particular actions of the drugs.

Figure 14 is a drawing showing chemical structures for specific serum cholesterol inhibitors mevastatin, fluvastatin, pravastatin, lovastatin and simvastatin. The inhibitors are HMG-CoA reductase inhibitors. Fluvastatin is an entirely synthetic

mevalonolactone derivative. Remaining reductase inhibitors are fungal compactin derivatives based on a hydronapthalene ring.

Figure 15A-B are drawings showing (15A) sphingmyelin and (15B) C-2 ceramide and dihydro-C-2 ceramide. The 3-hydroxyl group and 4, 5 trans carbon-carbon double bond in the sphingosine backbone are indicated by arrows.

DETAILED DESCRIPTION OF THE INVENTION

As discussed, the invention relates to anti-lipemic drugs and methods for using same to stabilize or reduce serum cholesterol level in a human patient or other subject mammal. Preferred anti-lipemic drugs generally include one identified effector of the SREBP-1 protein associated with one identified serum cholesterol inhibitor. More preferred are anti-lipemic drugs in which the effector and inhibitor components are specifically covalently linked together as a single formulation.

The term "anti-lipemic drug" is used herein to refer generically to a composition of this invention, preferably a specific synthetic or semi-synthetic drug, which has dual capacity to modulate serum cholesterol levels, ie, by modulating the LDL receptor and stabilizing or reducing serum cholesterol levels in the subject mammal. Preferred is an anti-lipemic drug with demonstrated capacity to increase LDL receptor levels and to reduce serum cholesterol levels as determined by specific in vitro and in vivo assays described below. As discussed below, capacity to reduce serum cholesterol levels by the inhibitor component is generally mediated by modulation of HMG CoA reductase, typically by inhibiting that enzyme sufficient to reduce serum cholesterol. As also discussed, the effector portion preferably increases production of the LDL receptor.

The anti-lipemic drugs disclosed herein can be made by recognized methods known in the field. For example, methods for making specific sphingolipids and especially ceramide and ceramide-related compounds have been disclosed in co-

pending U.S. Patent Application Serial. No. 08/998,262 entitled "Methods for Treatment of Conditions Associated with Lactosylceramide" filed on December 24, 1997, now issued as U.S. Patent 5,972,928 on October 26, 1999, the disclosure of which is incorporated herein by reference. See also Abe, A. et al., (1992) *J. Biochem.* 111:191-196; Inokuchi, J. et al. (1987) *J. Lipid Res.* 28:565-571; Shukla, A. et al. (1991) *J. Lipid Res.* 32:73; Vunnam, R.R. et al., (1980) *Chem. and Physics of Lipids* 26:265; Carson, K. et al., (1994) *Tetrahedron Lets.* 35:2659; and Akira, A. et al., (1995) *J. Lipid Research* 36:611.

More specific anti-lipemic drugs of this invention include as covalently linked components the effector and the serum cholesterol inhibitor. However for some applications other anti-lipemic drugs can be appropriate such as those including non-covalently linked components. Examples include those drugs provided as essentially co-administered formulations.

The molecular weight of a particular anti-lipemic drug will vary depending, e.g., on the specific SREBP-1 effector and serum cholesterol inhibitor chosen and the number of effectors and inhibitors making up the drug. However in most cases the anti-lipemic drug will have a molecular weight of less than about 10,000 kD to 35,000 kD particularly when the effector molecule is a protein or polypeptide sequence such as the N-SMase sequences or fragments thereof disclosed herein. Molecular weights will generally be significantly lower, e.g., between from about 100 kD to 1000 kD, preferably between from about 200 kD to 500 kD when the effector is a sphingomyelin or related molecule. Methods for determining the molecular weight are known and include standard molecular sizing methods such as SDS polyacrylamide gel electrophoresis.

Illustrative examples of specific anti-lipemic drugs in accord with this invention are shown in Figure 13. Figure 13 particularly shows use of combinations of SREBP-1 maturation upregulators (effectors) ceramide, N-SMase, and various lipid

lowering molecules; HMG CoA-reductase inhibitors (statins) in various human pathologies.

An "effector" of the LDL receptor and particularly the SREBP-1 protein is a molecule, usually an amino acid sequence, lipoprotein, lipid or like molecule with demonstrated capacity to modulate the LDL receptor and specifically maturation of the SREBP-1 protein as determined by the standard SREBP-1 maturation assay described below. Illustrative effectors are provided in the Examples and Figure 7.

A "serum cholesterol inhibitor" as that term is used herein generally refers to a recognized compound capable of reducing serum cholesterol levels in a subject mammal and especially a human patient. Preferred serum cholesterol inhibitors particularly interfere with cholesterol biosynthesis and especially HMG CoA-reductase activity, e.g., in the liver. More preferred serum cholesterol inhibitors are readily available commercially and include mevastatin, fluvastatin, pravastatin, lovastatin and simvastatin. See Figure 14 and the discussion below.

It has been unexpectedly found that TNF-α significantly stimulates maturation of SREBP-1 in cells through action of the N-SMase. That is, we have found that TNF-α is capable of inducing SREBP-1 maturation in a time and dose dependent manner. This induction was consistent with the kinetics of TNF-α mediated activation of neutral sphingomyelinase (N-SMase). Antibodies to N-SMase inhibited TNF-α induced SREBP-1 maturation suggesting that N-SMase is a necessary component of this signal transduction pathway. Ceramide, a product of sphingomyelin hydrolysis, was also found to be capable of inducing SREBP-1 maturation. Without wishing to be bound to theory, it appears that the mature form of SREBP-1 generated by TNF-α, sphingomyelinase or ceramide treatment translocates to the nucleus and binds the sterol regulatory element (SRE). This is believed to promote transcription of the gene upstream of the SRE. See Figure 7 for a schematic outline of these findings. It further appears that effectors of the SREBP-1 stimulate

the LDL receptor, particularly by enhancing SREBP-1 maturation, thereby stabilizing or reducing serum cholesterol in the subject mammal.

Therapeutic methods of the invention generally comprise administration of a therapeutically effective amount of at least one and typically one anti-lipemic drug as disclosed herein to a subject mammal such as a primate and especially a human patient in such treatment. The therapeutic treatment methods more specifically include administration of an effective amount of the anti-lipemic drug to a subject, particularly a mammal such as a human in need of such treatment for an indication disclosed herein.

Typical subjects of interest include those suffering from, suspected of suffering from, or susceptible to the conditions, disorders or diseases disclosed herein, e.g., hyperlipoproteinemia including hypercholesterolemia, stroke, obesity including compulsive eating disorders, cardiac disease including atherosclerosis, cerebral atherosclerosis, cholesteryl ester storage disorder, liver disease including organ transplantation failure and cirrhosis; diseases of the biliary system, and viral infection particularly those infections facilitating encephalitis or related disorders. More specific disclosure relating to these and other cholesterol related diseases including accepted methods for screening and diagnosing these disorders have been reported. See e.g., Brown, M.S. and Goldstein, J.L. (1993), *supra* and references cited therein.

A variety of specific anti-lipemic drugs can be employed in the present invention and particularly in the treatment methods described. Routine testing, e.g., in a standard in vitro assay optionally combined with another in vitro and/or in vivo assay, can in most instances readily identify suitable anti-lipemic drugs exhibiting desired selectivity and activity with respect to the target disorder or disease. As noted, preferred anti-lipemic drugs feature a specific effector of the SBREP-1 protein such as those effectors identified in the Examples including N-SMase or an effective fragment thereof; a sphingolipid and especially ceramide, a caspase, e.g., cpp32 protein

(caspase-3), or an effective fragment thereof; as well as other specific effectors discussed herein.

Additionally specific effectors are disclosed in the Examples and discussion which follows. For example, one anti-lipemic drug of this invention includes covalently linked in sequence: 1) an SREBP-1 effector comprising a chemically reactive group; and 2) a serum cholesterol inhibitor such as those disclosed herein including another chemically reactive group capable of specifically binding generally by covalent linkage to the reactive group of the effector. Optionally, the anti-lipemic drug further includes a bifunctional spacer, e.g., a heterobifunctional spacer, covalently linked between 1) and 2).

A more preferred anti-lipemic drug includes covalently linked in sequence: 1) a sphingolipid and especially sphingomyelin or ceramide; and 2) a specific serum cholesterol inhibitor as disclosed herein. In this embodiment, the ceramide is preferably naturally-occurring and can be any one of C-2, C-4, C-6 or C-8 ceramide. In embodiments in which the SREBP-1 effector is ceramide, the reactive group will typically be the C-3 group of ceramide. Preferred are serum cholesterol inhibitors that include a suitably chemically reactive hydroxyl (-OH) group, e.g., fluvastatin, simvastatin, lovastatin, pravastatin, mevinolin (compactin), or artorvastatin. Optionally, the anti-lipemic drug may include a bifunctional spacer covalently linked between 1) and 2), ie., providing a covalent bond between the C-3 group and the hydroxyl group.

Chemical structures for sphlingomyelin and specific ceramides (C-2 ceramide, dihydro-C-2-ceramide) are shown in Figures 15A and 15B.

Also preferred is an anti-lipemic drug that includes covalently linked in sequence: 1) the neutral sphingomyelinase (N-SMase) or the effective fragment thereof, and 2) a specific serum cholesterol inhibitor as disclosed herein. In this

embodiment in which the SREBP-1 effector is N-SMase or the fragment, the chemically reactive group will be a suitable amide bond. Preferred are serum cholesterol inhibitors that include a suitably chemically reactive hydroxyl (-OH) group, e.g., fluvastatin, simvastatin, lovastatin, pravastatin, mevinolin (compactin), or artorvastatin. Optionally, the anti-lipemic drug may include a bifunctional spacer and particularly a heterobifunctional spacer covalently linked between 1) and 2). Suitable linker sequences are known in the field and generally include chemically reactive groups on each end of a suitable polymeric sequence such as an amino acid sequence.

Illustrative N-SMase and fragments thereof for use in accord with this invention are provided in the examples and discussion which follow as well as the co-pending U.S. Application Serial No: 08/774,104 entitled "Recombinant N-SMases and Nucleic Acids Encoding Same" filed on December 24, 1996, now issued as U.S. Patent 5,919,687 on July 6, 1999, the disclosure of which is incorporated herein by reference.

In particular, a preferred neutral sphingomyelinase (N-SMase) is encoded by a sequence having at least 70%, 80%, 90% or 95% sequence identity to the sequence shown in Figure 12 (SEQ ID NO:1) or the complement thereof. A preferred fragment of the N-SMase includes a sequence having at least 70%, 80% or 90% sequence identity to nucleotides 862 to 1414 of SEQ ID NO:1 or the complement thereof. More specifically preferred is an N-SMase fragment that consists of nucleotides 862 to 1414 of SEQ ID NO:1 or the complement thereof. Methods for determining nucleotide sequence identity are known in the field and include use of well-known computer assisted programs such as FASTA and BLAST. See S. Altschul et al. *J. Mol. Biol.*, 215:403 (1990); and S. Altschul et al. *Nuc. Acids Res.*, 25: 3389-3402 (1997)for disclosure relating to the BLAST and related programs.

The term "effective fragments" as it relates to preferred N-SMase nucleotide fragments is used herein to refer to a specific nucleotides having significant activity in the standard in vitro SREBP-1 maturation assay described below. A specifically preferred example of an effective fragment of the N-SMase is nucleotides 862 to 1414 of SEQ ID NO:1.

As discussed, preferred anti-lipemic drugs of this invention exhibit significant activity in a standard SREBP-1 maturation assay. Preferably, the drug exhibits at least about 2 fold, preferably between about 2 to 10 fold, and more preferably from about 2 to 5 fold as determined by the assay. A preferred assay generally involves:

a) culturing suitable cells, e.g, HH-25 cells, in medium and adding the anti-lipemic drug for between from about 2 to 60 minutes, preferably between from about 10 to 30 minutes with about 15 minutes being generally preferred, typically followed by washing; and

b) detecting mature SREBP-1 (ie. proteolyically cleaved) and precursor SREBP-1 by performing Western immunoblotting with an anti-SREP-1 antibody such as those described below. In general, mass of the mature form of SREBP-1 can quantitatively determined vs. the precursor form. Presence of that mature form is indicative of SREBP-1 maturation and proteolysis. More specific methods for performing the assay are provided in the Examples which follow. Typically suitable control cells are included as a reference which cells are not exposed to the drug.

As also discussed, preferred anti-lipemic drugs of this invention exhibit good activity in a Northern blot assay for detecting and preferably quantifying LDL receptor mRNA. Additionally preferred anti-lipemic drugs are capable of increasing LDL receptor mRNA levels by at least about 10% and preferably at least from between about 20% to 50% as determined by the Northern blot assay or related mRNA detection assay. Methods for performing Northern blot assays are generally known and have been described, e.g, in Sambrook et al. in *Molecular Cloning: A*

Laboratory Manual (2d ed. 1989); and Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, 1989.

Suitable probes for detecting LDL mRNA are generally available and include cloned sequences of the human LDL receptor or related mammalian sequence available from Genbank. Information about Genbank can be obtain from the National Library of Medicine, 38A, 8N05, Rockville Pike, Bethesda, MD 20894. Genbank is also available on the internet at http :// www. ncbi .nlm. nih.gov. See generally Benson, D.A. et al. (1997) *Nucl. Acids. Res.* 25: 1 for a more complete description of Genbank.

An exemplary Northern blot assay for detecting and optionally quantitating LDL receptor mRNA levels is discussed below.

Preferred inhibitors of the HMG CoA reductase generally reduce or block synthesis of cholesterol in the liver, thereby facilitating compensatory reactions that can lead to a reduction in plasma LDL. A preferred assay for measuring this phenomenon is the standard HMG CoA reductase assay. As mentioned previously preferred anti-lipemic drugs of this invention exhibit an ID_{50} of between from about 20%, 30%, 40%, 50%, 60%, 70%, or 80% to about 90%, preferably between from about 30% to 50% as determined in the HMG CoA reductase assay. The standard HMG CoA reductase assay has been disclosed by Brown et al. (1978) *J. Biol. Chem.* 253: 1121. In this assay cultured human fibroblasts respond to an inhibition of the reductase by accumulating increased amounts of the enzyme when compared to a suitable control.

As also discussed additionally preferred anti-lipemic drugs are capable of reducing serum cholesterol as determined by a standard cholesterol assay. The drug preferably registers at least from about 5% or 10% to 20%, 30%, 40% or 50% decrease, preferably at least about 30% to 50% decrease as determined by the assay.

A preferred assay for measuring LDL cholesterol is commercially available from Sigma (St. Louis, Mo) and involves immunological separations. See also the National Cholesterol Education Program (NCEP) for information relating to acceptable cholesterol levels in humans.

A "high" or "high risk" cholesterol level or related term is defined herein as from between about 200 to 240 mg/dl (mM) cholesterol with a level greater than or equal to 240 mg/dl (mM) cholesterol being more generally understood to be indicative of high serum cholesterol. A normal serum cholesterol level is defined herein as being less than about 200 mg/dl (mM). For specific disclosure relating to conducting cholesterol tests see Brown, M.S. and Goldstein, J.L. *supra*, discussing the Guidelines of the NCEP Report of 1988.

Accordingly, "stabilization" or "reduction" of serum cholesterol as those terms are used herein will be understood to mean manifestation of a normal or near normal serum cholesterol level in the subject mammal. Also, a suitable control mammal in accord with this invention will preferably have a normal or near normal serum cholesterol level as determined by standard serum cholesterol tests.

Additional methods of this invention include modulating SREBP-1 levels in a mammal in which the method includes administering to the mammal a therapeutically effective amount of at least one and typically one of the anti-lipemic drugs disclosed herein. Typically, modulation of the SREBP-1 is evaluated by determining maturation of the protein as determined by the SREBP-1 maturation tests described in the Examples below.

The present invention also provides methods for modulating SREBP-1 levels in a mammal in which the method includes administering to the mammal a therapeutically effective amount of at least one and preferably one of the anti-lipemic drugs disclosed herein. In this embodiment, the SREBP-1 effector is neutral

sphingomyelinase (N-SMase) or a therapeutically effective fragment thereof; or a sphingolipid. As discussed, modulation of the SREBP-1 is typically evaluated by determining maturation of the protein as determined by the SREBP-1 maturation tests described in the Examples below. A preferred assay is the SREBP-1 proteolysis assay described below in the Examples.

Methods of this invention can be performed in vitro or in vivo using acceptable primary, cultured or immortalized cells such as those disclosed herein. Generally, these cells will be capable of exhibiting SREBP-1 maturation as defined herein including the HH-25 human hepatocytes described below. Methods for testing anti-lipemic drugs of interest and especially for use in human patient will preferably be conducted in vivo and may involve use of a suitable animal model depending on the method used. In this example, the model can be a suitable animal model such as those discussed previously. Alternatively, the methods can be performed on a suitable primate such as a human patient. Preferred is a human patient has been diagnosed as having, is suspected of having, or is susceptible to a cholesterol related disorder as defined above.

In embodiments in which the human patient is susceptible to one or more cholesterol related disorders, that susceptibility can be related to a genetic or environmental pre-disposition to the cholesterol related disorder. Methods for determining such pre-disposition are known in the field and include genetic testing. See Brown, M.S. and Goldstein, J.L. (1993) *supra*.

The invention thus provides methods for treating inappropriate (i.e. high) serum cholesterol levels as well as a disorder or condition associated therewith. In general, the methods include administration of a therapeutically effective amount of one or more anti-lipemic compounds of this invention to a subject mammal, particularly a human, suffering from or susceptible to the high serum cholesterol levels. Additionally contemplated is use of the present anti-lipemic compounds as

prophylactic drugs to prevent development of or reduce the severity of inappropriate serum cholesterol levels.

Compounds of the invention will be especially useful to a human patient who has or is suspected of having a cholesterol related disease, disorder or condition as defined herein. Compounds of the invention will be particularly useful in lowering serum cholesterol to normal or near normal levels in human patients. Specific examples of diseases which may be treated in accordance with the invention include hyperlipoporteinemia, stroke, cardiovascular disease and especially atherosclerosis as well as other specific disorders of conditions mentioned herein.

Without wishing to be bound by theory, it is believed the multiple and distinct covalently linked compounds of this invention (i.e. at least one identified anti-lipemic drug in combination with at least one identified SREP-1 effector) can significantly enhance efficacy of the anti-lipemic drug, e.g., by increasing synthesis of LDL receptor in subject cells.

Moreover, by virtue of the covalent linkage, the conjugates of the invention present the anti-lipemic drug and the SREP-1 effector to the subject cell essentially simultaneously, an effect that may not be readily achieved by administering the same compounds in a drug "cocktail" formulation without covalently linking the compounds.

It also has been reported that treatment with treatment with one drug can in turn sensitize a patient to another drug. Accordingly, the essentially simultaneous presentation to the subject cell of an anti-lipemic drug and SREP-1 effector via a conjugate of the invention may enhance drug activity, e.g., by providing synergistic results and/or by enhancing production of LDL receptors. Particular SREP-1 effectors of interest include sphingomyelin and especially ceramide and related compounds.

Also preferred is N-SMase as well as therapeutically effective fragments of that enzyme.

Administration of compounds of the invention may be made by a variety of suitable routes including oral, topical (including transdermal, buccal or sublingal), nasal and parenteral (including intraperitoneal, subcutaneous, intravenous, intradermal or intramuscular injection) with oral or parenteral being generally preferred. It also will be appreciated that the preferred method of administration and dosage amount may vary with, for example, the condition and age of the recipient.

Compounds of the invention may be used in therapy in conjunction with other medicaments such those with recognized pharmacological activity to lower concentrations of plasma lipoproteins. See Brown, M.S. and Goldstein, J.L. *supra.* Exemplary medicaments are recognized serum cholesterol inhibitors (i.e. reported to inhibit HMG CoA reductase) such as Lescol™ (fluvastatin from Sandoz Pharmaceuticals), Mevacor™ and Zocor™ (simvastatin and lovastatin, respectively, from Merck & Co.), Pravachol™ (pravastatin from Bristol-Myers Squibb Co.) and mevinolin (compactin).

The compounds of this invention may be used alone or in combination with other accepted anti-lipemic therapies including those implementing use of fibric acids, e.g., gembibrozil, clofibrate, fenofibrate, ciprofibrate or bezafibrate; bile acid-binding resins such as cholestyramine or colestipol; and probucol. The compounds of this invention can be administered before, during or after such therapies as needed.

While one or more compounds of the invention may be administered alone, they also may be present as part of a pharmaceutical composition in mixture with conventional excipient, i.e., pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, oral or other desired administration and which do not deleteriously react with the active compounds and are not deleterious to the

recipient thereof. Suitable pharmaceutically acceptable carriers include but are not limited to water, salt solutions, alcohol, vegetable oils, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, etc. The pharmaceutical preparations can be sterilized and if desired mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously react with the active compounds.

For parenteral application, particularly suitable are solutions, preferably oily or aqueous solutions as well as suspensions, emulsions, or implants, including suppositories. Ampules are convenient unit dosages.

For enteral application, particularly suitable are tablets, dragees or capsules having talc and/or carbohydrate carrier binder or the like, the carrier preferably being lactose and/or corn starch and/or potato starch. A syrup, elixir or the like can be used wherein a sweetened vehicle is employed. Sustained release compositions can be formulated including those wherein the active component is protected with differentially degradable coatings, e.g., by microencapsulation, multiple coatings, etc.

Therapeutic compounds of the invention also may be incorporated into liposomes. The incorporation can be carried out according to known liposome preparation procedures, e.g. sonication and extrusion. Suitable conventional methods of liposome preparation are also disclosed in e.g. A.D. Bangham et al., *J. Mol. Biol.*, 23:238-252 (1965); F. Olson et al., *Biochim. Biophys. Acta*, 557:9-23 (1979); F. Szoka et al., *Proc. Nat. Acad. Sci.*, 75:4194-4198 (1978); S. Kim et al., *Biochim. Biophys. Acta*, 728:339-348 (1983); and Mayer et al., *Biochim. Biophys. Acta*, 858:161-168 (1986).

The liposome may be made from one or more of the conjugates discussed above alone, or more preferably, in combination with any of the conventional synthetic or natural phospholipid liposome materials including phospholipids from natural sources such as egg, plant or animal sources such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, sphingomyelin, phosphatidylserine or phosphatidylinositol. Synthetic phospholipids also may be used e.g., dimyristoylphosphatidylcholine, dioleoylphosphatidylcholine, dioleoylphosphatidycholine and corresponding synthetic phosphatidylethanolamines and phosphatidylglycerols. Cholesterol or other sterols, cholesterol hemisuccinate, glycolipids, 1,2-bis(oleoyloxy)-3-(trimethyl ammonio)propane (DOTAP), N-[1-(2,3-dioleoyl)propyl]-N,N,N-trimethylammonium chloride (DOTMA), and other cationic lipids may be incorporated into the liposomes. The relative amounts of the one or more compounds and additives used in the liposomes may vary relatively widely. Liposomes of the invention suitably contain about 60 to 90 mole percent of natural or synthetic phospholipid; cholesterol, cholesterol hemisuccinate, fatty acids or cationic lipids may be used in amounts ranging from 0 to 50 mole percent; and the one or more therapeutic compounds of the invention may be suitably present in amounts of from about 0.01 to about 50 mole percent.

It will be appreciated that the actual preferred amounts of active compounds used in a given therapy will vary according to the specific compound being utilized, the particular compositions formulated, the mode of application, the particular site of administration, etc. Optimal administration rates for a given protocol of administration can be readily ascertained by those skilled in the art using conventional dosage determination tests conducted with regard to the foregoing guidelines.

In general, for treatment of a lipid related disease as disclosed herein and particularly hyperlipoproteinemia, stroke, coronary heart disease and especially atherosclerosis, a suitable effective dose of one or more compounds of this invention will be in the range of from 0.01 to 100 milligrams per kilogram of bodyweight of

recipient per day, preferably in the range of from 0.1 to 50 milligrams per kilogram bodyweight of recipient per day, more preferably in the range of 1 to 20 milligrams per kilogram bodyweight of recipient per day. The desired dose is suitably administered once daily, or several sub-doses, e.g. 2 to 5 sub-doses, are administered at appropriate intervals through the day, or other appropriate schedule.

A preferred dose for many compounds of this invention will be in the range of those dosages accepted for identified HMG CoA reductase inhibitors with lower than that range being preferred for many patients. See the *Physicians' Desk Reference, supra* for more specific information relating to recommended doses for HMG CoA reductase inhibitors with anti-lipemic activity.

In another aspect, the invention also provides methods for detecting an effector of the sterol regulatory element binding protein-1 (SREBP-1). In one embodiment, the method includes the steps of:

a) providing a population of cells capable of expressing SREBP-1,

b) contacting the cells with a candidate effector in an amount sufficient to induce maturation of the SREBP-1,

c) culturing the cells in medium; and

d) detecting maturation of the SREBP-1 as indicative of the effector of the SREBP-1.

Illustrative effectors for use in the method are include those specifically described in the Examples and Figure 7, e.g.,. tumor necrosis factor (TNF-α), neutral sphingomyelinase (N-SMase) or an effective fragment thereof, sphinogmyelin, ceramide, cpp32, or cholesterol. See also Figure 13.

The invention also includes a method for detecting an effector of LDL receptor biosynthesis. In one embodiment, the method includes:

a) providing a population of cells responsive to ceramide and capable of expressing SREBP-1,

b) contacting the cells with a candidate effector in an amount sufficient to induce maturation of the SREBP-1,

c) culturing the cells in medium; and

d) detecting biosynthesis of the LDL receptor as being indicative of the effector of the LDL receptor.

In one embodiment of the method, illustrative candidate effectors of the LDL receptor is tumor necrosis factor (TNF-α), neutral sphingomyelinase (N-SMase) or an effective fragment thereof; sphinogmyelin, ceramide, cpp32, or cholesterol.

Also provided by the present invention is a method for determining therapeutic capacity of an effector of SREBP-1 for treating a cholesterol related disease in a mammal. In one embodiment, the method includes:

a) providing a population of cells capable of expressing SREBP-1,

b) contacting the cells with a candidate compound in an amount sufficient to induce maturation of the SREBP-1,

c) culturing the cells in medium; and

d) detecting maturation of the SREBP-1 as indicative of the therapeutic capacity of the effector in treating the disease.

The present invention also provides methods for determining therapeutic capacity of any one of the anti-lipemic drugs disclosed herein for treating a cholesterol related disease in a mammal. In one embodiment, the method includes:

a) providing a population of cells capable of expressing SREBP-1,

b) contacting the cells with the anti-lipemic drug in an amount sufficient to induce maturation of the SREBP-1,

c) culturing the cells in medium; and

d) detecting maturation of the SREBP-1 as indicative of the therapeutic capacity of the anti-lipemic drug in treating the disease.

Also provided herein are methods for determining therapeutic capacity of one or more of the anti-lipemic drugs disclosed herein using a Watanabe heritable hyperlipidemic rabbit or apolipoprotein and negative mouse as an animal model. In one embodiment, the method includes:

a) administering at least one of the anti-lipemic drugs to the rabbit or mouse in an amount sufficient to reduce serum cholesterol levels by at least from about 10 to 20% as determined by a standard cholesterol assay; and

b) detecting the serum cholesterol reduction in the rabbit or mouse as being indicative of the therapeutic capacity of the anti-lipemic drug to treat the cholesterol related disease.

Methods of this invention can optionally include monitoring LDL receptor activity as being indicative of the effector of the SREBP-1. In this embodiment, the receptor activity can be suitably monitored and quantified if desired by one or a combination of standard strategies. For example, a variety of specific methods have been reported to monitor LDL receptor activity and particularly to detect increases or decreases in the level of LDL receptors. See Brown, M.S. and Goldstein, J.L. (1993), *supra* and references cited therein for several immunological and molecular approaches. A preferred method is the standard LDL receptor Northern blot assay disclosed herein.

Suitable cells for use in the methods of this invention are described in the Examples which follow.

Preferred are cells which include SREBP-1 and are capable of SREBP-1 maturation as determined by the standard assay described herein. More preferred are cells responsive to an increase or decrease in intracellular sphingolipid and especially

ceramide or a related compound such as human hepatocytes as provided in the Examples below.

Suitable effectors or candidate compounds for use with the methods can be those specific compounds described herein neutral sphingomyelinase (N-SMase) or an effective fragment thereof; sphinogmyelin, ceramide, cpp32, or cholesterol. An illustrative neutral sphingomyelinase (N-SMase) is encoded by a sequence having at least 70%, 80%, or 90% sequence identity to the sequence represented by SEQ ID NO:1 or complement thereof. Alternatively, the effective fragment of the neutral sphingomyelinase (N-SMase) can include a sequence having at least 70%, 80% or 90% sequence identity to nucleotides 862 to 1414 of SEQ ID NO:1 or complement thereof.

It is preferred that the anti-lipemic drugs as well as components thereof (e.g., ceramide) be substantially pure. That is, the drugs will be present in at least 90 to 95% homogeneity (w/w). Anti-lipemic drugs having at least 98 to 99% homogeneity (w/w) are most preferred for many pharmaceutical, clinical and research applications. Once substantially purified the drug should be substantially free of contaminants for therapeutic applications. Once purified partially or to substantial purity, the drugs can be used therapeutically, or in performing preferred *in vitro* or *in vivo* assays as disclosed herein. Substantial purity can be determined by a variety of standard techniques such as chromatography and gel electrophoresis.

The Examples 1-8 below illustrate that TNF-α is capable of inducing SREBP-1 proteolysis independent of the presence of sterols. Exogenously supplied sphingomyelinase and ceramide are also capable of inducing SREBP-1 proteolysis in a time and dose dependent manner. The kinetics of SREBP-1 maturation is consistent with those of neutral sphingomyelinase activation by TNF-α. Further, SREBP-1 maturation can be blocked with anti-N-SMase antibodies indicating that neutral sphingomyelinase is necessary for TNF-α induced, sterol independent SREBP-1

cleavage. The product of sterol independent SREBP-1 proteolysis is capable of nuclear translocation and binds to the sterol regulatory element.

All documents mentioned herein are incorporated herein by reference.

The following abbreviations are used throughout this disclosure including the following examples as needed: N-SMase, neutral sphingomyelinase; LDLr, Low Density Lipoprotein receptor; SREBP-1, Sterol Regulatory Element Binding Protein-1. Numbered citations are listed in numerical order below.

Example 1- The effect of TNF-α on Neutral sphingomyelinase activity

Neutral sphingomyelinase activity increased rapidly with the addition of TNF-α. See Figure 1. A maximal 2.5 fold increase in activity was observed 15 minutes after TNF-α was added to the cells. The gradual return of N-SMase activity to control levels within 1 hour contrasted the rapid onset of activation and is reflected in the asymmetric kinetic profile observed.

Figure 1 illustrates the effect of TNF-α on neutral sphingomyelinase activity and is explained in more detail as follows: Confluent cultures of HH-25 cells were washed once with PBS and incubated in serum free media for 30 minutes prior to the addition of TNF-α (10ng/ml). At the indicated time, cells were harvested in PBS, pelleted and frozen. Cells were subsequently lysed as described in materials and methods. N-SMase assays were performed in duplicate as described. Error bars represent ± one standard deviation from the mean.

Example 2 Kinetics of SREBP-1 proteolysis

Sterol independent SREBP-1 maturation in response to TNF-α closely paralleled the kinetics of TNF-α induced N-SMase activation. The mass of the mature form of SREBP-1 was found to increase 2 fold after 5 minutes and 3 fold after 15 minutes of incubation with TNF-α. See Figure 2A. The amount of mature

SREBP-1 returned to control levels within one hour. This effect could not be recapitulated with EGF or PDGF treatment. The increase in mature SREBP-1 levels was accompanied by a concomitant decrease in the intensity of the band corresponding to the precursor form of SREBP-1. See Figure 2B. After 60 minutes of treatment significantly less precursor SREBP-1 was visible.

To incorporate the observed increase in mature SREBP-1 and the concomitant decrease in precursor SREBP-1 into a single variable, the ratio of precursor SREBP-1 to mature was plotted. See Figure 2B. A maximal 1.5 fold decrease in the precursor to mature ratio occurred 45 minutes after TNF-α was added to the media. The decrease in precursor to mature ratio was more pronounced in the initial 30 minutes of treatment. This is also consistent with the kinetics of TNF-α induced N-SMase activation.

To explore the possibility that plasma membrane sphingomyelinase was involved in the signal transduction pathway leading to SREBP-1 proteolysis, cells were treated with exogenously supplied bacterial sphingomyelinase. Sphingomyelinase induced a dramatic change in the relative amounts of precursor and mature SREBP-1. As seen in Figures 2A-2B, a 2.5 fold increase in mature SREBP-1 levels was observed after 15 minutes treatment. Unlike TNF-α, the increase in mature SREBP-1 induced by sphingomyelinase persisted after 60 minutes. Sphingomyelinase was also capable of reducing the level of the precursor form of SREBP-1. See Figures 2A-B. Treatment with purified recombinant human sphingomyelinase produced similar results.

Much of the signal transducing ability of N-SMase has been ascribed to its ability to generate the lipid second messenger ceramide. Accordingly, the ability of a cell permeable ceramide analog C_2-ceramide (N-acetylsphingosine) was tested to induce SREBP-1 maturation. C_2-ceramide also induces SREBP-1 maturation in a sterol independent manner with greater magnitude than what was observed with either

TNF-α or sphingomyelinase. C_2-ceramide increased the level of mature SREBP-14 fold after 30 minutes of treatment . See Figures 2A-2B. The persistent elevation of mature SREBP-1 levels observed with sphingomyelinase treatment also accompanied C_2-ceramide treatment. The increase in mature SREBP-1 is recapitulated with the addition of bovine brain ceramides but could not be induced with C_2-dihydroceramide, PL-A_2, or Phospholipid D treatment .

The kinetics of SREBP-1 maturation presented in this example would suggest that SREBP-1 proteolysis is a sufficiently early event to be involved in providing cholesterol to apoptotic cells. However, there was no evidence of apoptosis in the HH-25 human hepatocyte cell line used in this study. Without wishing to be bound to theory, it is conceivable that the sterol independent induction of SREBP-1 maturation in hepatocytes is a physiologic process that does not require that apoptosis be induced. Alternatively, the two pathways may diverge before the cell has been committed to apoptosis suggesting a manner in which sterol independent SREBP-1 proteolysis could be employed independent of the induction of apoptosis.

The sterol-independent cleavage of SREBP-1 observed with human hepatocytes could also occur by ceramide generated by the TNF-α induced N-SMase activation. This phenomenon may be reconstituted by the exogenous addition of N-SMase and/or C_2 ceramide to the hepatocytes.

Figures 2A-2C illustrate effects of TNF-α sphingomyelinase and C_2-ceramide on the kinetics of SREBP-1 maturation- Figures 2A-2C is explained in more detail as follows: Cells were maintained in media supplemented with1 μg/ml 25-hydroxy cholesterol and 15 μg/ml cholesterol for 24 hours before the experiment. The cells were treated for the indicated time as described in materials and methods. The cells were then harvested in PBS, pelleted and frozen. Lysis and nuclear fractionation were performed as described. Nuclear fractions (50μg of protein) were electrophoresed on a 7.5% polyacrylamide gel and transferred to a PVDF membrane. Western blotting

was performed as described. Band intensity was quantified via densitometry. Error bars represent ± one standard deviation from the mean. 2A) The kinetics of SREBP maturation as measured by the increase in mature SREBP-1 are plotted. Fold increase was calculated by comparing each time point to the corresponding control value (TNF-α is represented by stippled bars, bacterial sphingomyelinase is represented by light gray bars and C_2-ceramide by the dark bars.) 2B) Cells were treated with TNF-α (10ng/ml) and prepared as described above. The bands corresponding to the precursor and mature forms of SREBP-1 were quantified and their ratio plotted. 2C) Representative Western blots from which numerical data was derived. Incubation time is indicated above and applies to all conditions. The membranes were exposed to film for 15 seconds. P and M denote the precursor and mature forms of SREBP-1 respectively.

Example 3- Effects of TNF-α, sphingomyelinase and C_2-ceramide on apoptosis in hepatocytes

To demonstrate that the observed maturation of SREBP-1 was not an artifact of the more general phenomenon of apoptosis induced proteolysis we performed DNA laddering assays. The 160 bp DNA ladder characteristic of cells undergoing apoptosis was not observed in any of the samples .

TNF-α, C_2-ceramide and sphingomyelinase did not induce apoptosis demonstrating that in hepatocytes, SREBP-1 maturation is not part of the more general phenomenon of apoptotic protein hydrolysis.

Example 4- Effects of TNF-α, sphingomyelinase and C_2-ceramide concentration on SREBP-1 maturation

The extent of TNF-α induced SREBP-1 maturation did not vary appreciably with concentration. A maximal effect was observed with 10ng/ml of TNF-α. See Figures 3A-C. 250 milliunits of sphingomyelinase activity induced an 80% decrease in the precursor to mature ratio. As little as 1μM of C_2-ceramide was effective in

producing an 81% maximal effect. The maximal effect however, was obtained with a C_2-ceramide concentration of 50 M. See Figures 3A-C.

Figures 3A-3C show effects of TNF-α, sphingomyelinase and C_2-ceramide concentration on SREBP-1 maturation- The Figures 3A-3C are explained in more detail as follows. Cells were treated with either TNF-α, sphingomyelinase or C_2-ceramide at the indicated concentrations. Nuclear pellets were prepared and electrophoresed (50μg of protein). The bands corresponding to the precursor and mature froms of SREBP-1 were quantified. The precursor to mature ratios were normalized to a single control to facilitate comparison. The control ratio was arbitrarily assigned a value of 1. A Unit of sphingomyelinase activity hydrolyzes 1.0 μmol of sphingomyelin per minute at 37°C. Figure 3A(ng/ml TNF-α); Figure 3B (mUnits of sphingomyelinase); Figure 3C (micromolar C2-ceramide)

Example 5- The effect of anti-N-SMase antibodies on TNF-α mediated SREBP-1 maturation

The availability of anti-N-SMase antibodies allowed us to examine the effects of TNF-α on this pathway independent of N-SMase activation (10). Polyclonal anti-N-SMase antibodies at a dilution of 1:200 completely block TNF-α induced SREBP-1 maturation. See Figure 4. The suppression of TNF-α mediated SREBP-1 maturation was relieved with increasing antibody dilution. Preincubation with preimmune serum at the same dilution had no appreciable effect.

This example shows that pre incubation with anti-N-SMase antibody effectively blocked TNF-α induced SREBP-1 maturation. Inhibition was not observed with pre-immune serum treatment and was relieved with increasing antibody dilution. Such findings are confirmed by other studies such as those showing the ability of the antibody to inhibit TNF-α induced increases in cholesterol ester synthesis and N-SMase induced increases in $[^{125}]$I-LDL binding, internalization and degradation in human fibroblasts (15, 16).

Figure 4 shows effect of anti-N-SMase antibodies on TNF-α induced SREBP-1 maturation. The Figure 4 is explained in more detail as follows. Cells were maintained in media supplemented with 1 μg/ml 25-hydroxycholesterol and 15 g/ml cholesterol for 24 hours before the experiment. The cells were switched to serum free media for 15 minutes and then incubated with anti-N-SMase antibodies or rabbit preimmune serum at the indicated dilution for 30 minutes prior to TNF-α addition (10ng/ml). The cells were then harvested, pelleted and lysed as described. The samples were electrophoresed on a 7.5% polyacrylamide gel and transferred to a PVDF membrane. Bands were visualized as described. Film was exposed for 15 seconds.

Example 6- Effects of TNF-α, C_2-ceramide and Sphingomyelinase on the subcellular localization of SREBP-1

To determine if the SREBP-1 fragment generated by TNF-α, C_2-ceramide or sphingomyelinase treatment was capable of nuclear translocation, immunofluorescence studies were persued. Previous immunofluorescence studies have relied on the overexpression of precursor and mature forms of SREBP-1 (14). We were able to visualize endogenous SREBP-1 in treated and untreated cells with polyclonal antibodies directed against the DNA binding domain of SREBP-1. Since the DNA binding domain is common to both the precursor and mature forms, examination of the total distribution of endogenous SREBP-1.was possible.

TNF-α, C_2-ceramide and sphingomyelinase all are capable of inducing changes in the subcellular localization of SREBP-1. See Figure 5A. Untreated cells display an even staining pattern throughout their cell bodies. This is consistent with the localization of precursor SREBP-1 to intracellular membranes (14). However, cells treated with TNF-α, C_2-ceramide or sphingomyelinase all exhibit intense nuclear staining and little extra-nuclear staining. See Figures 5B-5D. The rapid change in the subcellular localization of SREBP-1 is consistent with a

precursor/product relationship between the two forms and provides additional evidence that the mature SREBP-1 fragment generated by treatment is capable of nuclear translocation.

Figures 5A-5D show indirect immunofluorescence of SREBP-1. Figures 5A-5D are discussed in more detail as follows. SREBP-1 was visualized with rabbit polyclonal antibodies directed towards the N-terminal DNA binding domain which is common to both the precursor and mature forms. Cells were maintained in media supplemented with 1μg/ml 25-hydroxycholesterol and 15 μg/ml cholesterol for 24 hours before the experiment. Immunofluorescence was performed as described. All magnifications are 40X and all photographs were taken of samples that were fixed 30 minutes after initiating treatment. Figure 5A) Control cells, Figure 5B) Cells treated with TNF-α (10ng/ml), Figure 5C) Cells treated with sphingomyelinase (100mUnits), Figure 5D) Cells treated with C_2-ceramide (10μM).

Example 7-Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were pursued to demonstrate that the mature SREBP-1 fragment is additionally capable of binding to its consensus sequence. The amount of electorphoretically retarded probe increases with time following TNF-α treatment. See Figure 6A. The kinetics of this process is consistent with the activation of N-SMase. The amount of probe bound increases with sphingomyelinase and ceramide treatment. As expected, C_2-ceramide induces a more rapid accumulation of active, nuclear SREBP-1 than either TNF-α or sphingomyelinase. See Figures 6A-6C. Antibodies directed towards the DNA binding domain of SREBP successfully compete with the oligonucleotide probe for binding. See Figure 6D. Binding of the probe is not titrated by an unrelated oligonucleotide but is decreased with the addition of a non-radioactive competing probe .

Figures 6A-6D show electrophoretic mobility shift assays. Figures 6A-D are explained in more detail as follows. Cells were maintained in sterol supplemented media. Nuclear pellets were prepared and assayed as described in materials and methods. Probe that has been bound by mature SREBP-1 is indicated as "Bound". Unbound probe is indicated as "Free". The kinetics (in minutes) of SREBP-1 binding to the probe in response to treatment with (Figure 6A). TNF-α (10ng/ml), (Figure 6B) sphingomyelinase (100mUnits) and (Figure 6C) C_2-ceramide (10 M). (Figure 6D). The cells were treated with either TNF-α (10ng/ml), sphingomyelinase (100mUnits) or C_2-ceramide (10μM) for 15 minutes. Supershift assays were then performed with antibodies raised against the DNA binding domain of SREBP-1. The presence or absence of antibody is indicated by (+) and (-) respectively. Pre-immune IgG was used as a control.

The gel mobility shift experiments in Figures 6A-D clearly indicate that TNF-α, N-SMase and C_2 ceramide all induce SREBP-1 levels in hepatocytes. It is known that TNF-α induces sterol metabolism in cultured human fibroblasts (15) and LDL receptors (16, 17). The present data indicate that indeed TNF-α induces LDL receptor mRNA level in human hepatocytes. One result is that TNF-α induced increase in mature SREBP-1 level is accompanied by increased LDL receptors and sterol metabolism.

Example 8- Effects of Overexpression of Neutral sphingomyelinase (N-SMase) and Recombinant N-SMase on the Maturation of Sterol-regulatory element binding protein-1 and Low density Lipoprotein Receptor Expression in cultured Human Hepatocytes

The present example was conducted to address whether the overexpression of N-SMase employing two separate N-SMase plasmid DNA(PHH-1,representing the entire nucleotide sequence in N-SMase cDNA and PHH-11, representing nucleotide sequence 862-1414) would increase the maturation of SREBP-1 and LDL receptor

mRNA expression in a human hepatocyte cell line HH-11. Cells transfected with mock plasmid cDNA(PSV-SPOT) served as a control and cells incubated with C-2 ceramide previously shown to induce SREBP-1 maturation served as a positive control.

Briefly, human hepatocytes($1x10^4$) were seeded in sterile 100 mm^2 in medium containing 10% dialyzed, heat inactivated fetal bovine serum without antibiotics. Twenty four hours later medium was replaced with 9 ml of fresh serum free medium. After incubation for 30 min at 37°C 5-40 μg of the plasmid DNA in 1 ml of a $CaCl_2$ solution (mixed with equal volume of 0.25-2.5 M $CaCl_2$ solution in HEPES buffer and HEPES buffer pH 6.95). Following gentle mixing incubation of cells was continued for 5-24 hr at 37 °C. The transfection reaction was terminated by removing the medium and washing the cells with serum free medium. Next, fresh serum supplemented medium was added and incubation was continued for an additional 24 hr and cells were harvested in appropriate buffer centrifuged and stored frozen until further analysis. Cell pellets were homogenized and suitable aliquots subjected to Western immunoblot analysis as described below and in Examples 1-7 above. Total RNA was isolated from another batch of cells transfected as above and subjected to Northern analysis employing a ^{32}P labeled LDL receptor consensus sequence. The autoradiographs were developed and photographed.

Cells transfected with 0.2μg/ ml of PHH1 or PHH11 showed a 2-fold increase in N-SMase activity compared to mock cDNA transfected cells. This was accompanied with aPHH1and PHH11 concentration dependent increase in the maturation of SREBP-1 in human hepatocytes. See Figure 7. As shown in lanes 3-6 transfection of cells with 5,10,20,40 μg of PHH1 plasmid DNA/dish resulted in a gradual but marked increase in the maturation of SREBP-1as compared to mock cDNA transfected cells (lane1,Figure 7). In contrast, a marked increase in the maturation of SREBP-1 was noted in cells transfected with 20μg/dish of PHH11 plasmid DNA (lane 9 Figure 7) but subsided at a higher concentration. As expected

form the Examples 1-7 above, cells incubated with C-2 ceramide (μM) markedly increased the maturation of SREBP-1(lane 2 Figure 7). In additional experiments we observed that increasing the time of transfection from 8 hr to 24 hr decreased the maturation of SREBP-1 in hepatocytes. Moreover, decreasing the concentration of $CaCl_2$ from 2.5M to 0.25M was ineffective.

Northern gel analysis revealed that transfection with PHH1 and PHH11(lanes 2, 3, respectively in Figure 8) significantly increased the level of LDL receptor mRNA as compared to cells transfected with mock cDNA (lane 1 Figure 8).

In another experiment hepatocytes were incubated with purified bacterial recombinant N-SMase. Preferred methods of making and using the recombinant N-SMase are described in the co-pending U.S. Patent Application Serial No.08/774,104, now issued as U.S. Patent 5,919,687. That N-SMase was subjected to western immunoblot analysis employing antibody against SREBP-1. As shown in Figure 9, cells incubated with C-2 ceramide markedly increased the maturation of SREBP-1 (lane1). In comparison the r-N-SMase exerted a concentration –dependent increase in the maturation of SREBP-1 (lane 2, 3, 4, 5 representing 0.4, 0.8, 2,and 4 μg/ml of r-N-SMase, respectively).

This example shows that overexpression of N-SMase or feeding r-N-SMase to hepatocytes stimulates the maturation of SREBP-1 and consequently an increase in the LDL receptor mRNA levels.

The Examples 1-8 above highlight a novel pathway by which SREBP-1 maturation could be effected in a sterol independent manner. It was found that TNF-α is capable of inducing SREBP-1 maturation in a sterol independent manner in human hepatocytes. These findings are not a general response to growth factors, as they could not be recapitulated with EGF or PDGF. The maturation, nuclear translocation, and SRE binding activity of SREBP-1 in response to TNF-α closely paralleled the

kinetics of N-SMase activation. The effect of TNF-α on SREBP-1 maturation could be reconstituted with exogenously supplied bacterial or human sphingomyelinase C_2-ceramide but could not be recapitulated with dihydroceramide, PL-A_2, or PL-D.

In particular, Examples 1-7 show that addition of C_2-ceramide, a water soluble ceramide analog, or bacterial sphingomyelinase mimicked the effect of TNF-α on SREBP-1 maturation. C_2-ceramide and sphingomyelinase induced more extensive SREBP-1 maturation than TNF-α. Without wishing to be bound to theory, this observation may reflect the presence of a regulatory event upstream of ceramide generation that is effectively bypassed with exogenous ceramide or sphingomyelinase. Also, the lack of apparent dose dependence observed with TNF-α treatment might be attributable to saturable binding of the TNF-α receptors or an internal regulatory event that reduces the signaling capacity of the TNF-α receptors.

The present data and discussion indicate a model in which TNF-α initiates SREBP-1 proteolysis. The model (Figure 7) in which there is shown TNF-α binding to one or more of its cell surface receptors and in so doing promotes the activation of N-SMase. N-SMase hydrolyzes membrane sphingomyelin into ceramide and phosphocholine. Ceramide, in turn, activates a protease perhaps CPP32 that mediates SREBP-1 maturation. According to the model, the mature SREBP-1 then migrates into the nucleus as shown and drives the transcription of genes with an upstream sterol regulatory element.

The model illustrated in Figure 7 clarifies how sterol homeostasis can occur in the presence of increased cytosolic sterols, which would be predicted to suppress SREBP-1 maturation. One advantage conferred by the participation of neutral sphingomyelinase in cholesterol homeostasis is that it is capable of providing a short term solution to cholesterol starvation through mobilization of plasma membrane cholesterol and can facilitate long term compensatory mechanisms by promoting the maturation of SREBP-1.

The model shown in Figure 7 also shows that TNF-α is capable of inducing SREBP-1 proteolysis independent of the presence of sterols. Exogenously supplied sphingomyelinase and ceramide are also capable of inducing SREBP-1 proteolysis in a time and dose dependent manner. The kinetics of SREBP-1 maturation is consistent with the activation of neutral sphingomyelinase by TNF-α. Furthermore, recombinant human N-SMase can also exert a time and concentration dependent induction of SREBP-1 maturation. In addition, anti-N-SMase antibodies block SREBP-1 maturation. These findings indicate that neutral sphingomyelinase is necessary for TNF-α induced, sterol independent SREBP-1 cleavage.

The present examples and discussion identify N-SMase in the TNF-α initiated signal transduction pathway leading to SREBP-1 maturation and provide evidence that ceramide is the second messenger employed. Also shown is an important role for TNF-α in the regulation of cholesterol homeostasis.

The present findings are summarized as follows. The role of TNF-α as a mediator of SREBP-1 maturation was investigated in human hepatocytes.

One significant aspect of the above Examples and discussion is that ceramide stimulated SREBP-1 maturation even in the presence of cholesterol and 25-hydroxycholesterol both of which are known suppressers of SREBP-1 maturation. This indicates that ceramide mediated maturation of SREBP-1 maturation is a novel, sterol independent mechanism by which cholesterol homeostasis may be regulated.

The following materials and methods were used as needed in the above Examples 1-8.

1. *Materials*- A continuous line of human hepatocytes designated HH-25 were prepared from normal human tissue (18). Alpha modified minimal essential

medium was purchased from Mediatech (Herndon, VA). Fetal bovine serum was purchased from Hyclone, Salt Lake City, Utah. F10 media and the insulin-transferrin-selenium supplement were purchased from Gibco-BRL (Gaithersburg, MD). Human recombinant EGF, PDGF and TNF-α were from Upstate Biotechnology (Lake Placid, NY). C_2-ceramide (N-acetylsphingosine) was obtained from Matreya (Pleasant Gap, PA). [^{14}C]-sphingomyelin (specific activity 50mCi/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). Anti-SREBP-1 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Sphingomyelinase from Streptomyces species and all other redrugs were obtained from Sigma.

2. *Cell Culture*- HH-25 cells were grown in alpha-minimal essential media supplemented with 100units/ml penicillin, 100 g/ml streptomycin, 10 g/ml insulin, 0.1μM selenium, 5.5 μg/ml transferrin, 0.5 μg/ml linoleic acid and 10% fetal bovine serum (media A). The cells were incubated in serum free F10 media for 30 to 60 minutes prior to initiating treatment with TNF-α, C_2-ceramide or sphingomyelinase.

3. *Cell fractionation*- Following treatment, the cells were washed with 5ml of PBS and pelleted at 1500xg for 10 minutes at 4°C. The pellet was stored at -70°C and lysed in 0.5ml buffer A (10mM HEPES pH 7.4, 5mM EDTA, 0.25mM EGTA, 50mM NaF, 7mM β-mercaptoethanol, 0.35M sucrose, 0.1% NP-40 and protease inhibitors 1mM PMSF, 2μg/ml aprotinin, 10μg/ml leupeptin and 5μg/ml pepstatin). The lysate was centrifuged at 12,000 x g for 15 minutes at 4°C to prepare a nuclear fraction. The protein concentration of these samples was determined by the method of Lowry et al. al. (19).

4. *Neutral Sphingomyelinase Assay*-After stimulation with TNF-α for the indicated time intervals, the cells were washed once with 5ml PBS and harvested. The pellet was stored frozen at -70°C and resuspended in 0.5ml buffer B (100mM Tris HCl pH 7.4, 0.1% triton X-100, 1mM EDTA and protease inhibitors). The cell

suspension was sonicated 3 times (3 second bursts) using a probe sonicator and centrifuged at 500xg at 4°C for 5 minutes. The supernatant was used as the enzyme source.

100 μg of protein was assayed for neutral sphingomyelinase activity in a buffer consisting of 50mM Tris HCl pH 7.4, 0.1% triton X-100, 0.1mg BSA, 5mM $MgCl_2$, and 50 moles [^{14}C] sphingomyelin (12,000 dpm). The assay was incubated at 37°C for 1.5 hours and terminated with the addition of 1ml of 10% TCA. The precipitate was pelleted (1000xg at 4°C for 20 minutes) and 1ml of the supernatant was extracted with 1 ml anhydrous diethyl ether. 0.5ml of the aqueous phase was removed for liquid scintillation counting.

5. *Immunoblotting*- 50μg of nuclear protein was separated by gel electrophoresis on a 7.5% polyacrylamide gel. Gels were calibrated by high range molecular weight markers (Bio-Rad product #161-0303, New York, NY) which were transferred to a polyvinyl diflouride (PVDF) membrane and visualized with coomassie staining. Rabbit polyclonal antibodies against SREBP-1 were used at 0.5 μg/ml according to the instructions of the manufacturer. The antibody was visualized with horseradish peroxidase conjugated anti-rabbit IgG made in donkey (Amersham) using the Enhanced Chemiluminescence (ECL) Western Blotting Detection System Kit (Amersham). PVDF membranes were exposed to Hyperfilm ECL (Amersham) for the indicated time. Immunoblots were quantified via densitometry performed on a PDI densitometer scanner (model 20J7) coupled to a SPARC IRC workstation.

5. *Indirect Immunofluorescence*-Cultured HH-25 cells were grown on coverslips and treated as described. After treatment, the cells were washed 3 X 5 minutes with PBS containing 1mM $MgCl_2$ and 0.1mM $CaCl_2$ (solution A). The cells were fixed with 3% paraformaldehyde in solution A for 10 minutes and permeabilized with 0.5% Triton X-100 in solution A for 6 minutes at room temperature. The coverslips were then washed 3 x 5 minutes with solution A.

Primary antibody (anti-SREBP1) was used at a dilution of 0.5 g/ml in PBS and applied for 1 hour with gentle shaking. The cells were washed as above and a FITC conjugated anti-rabbit IgG secondary antibody, was applied for 1/2 hour according to the instructions of the manufacturer. The coverslips were washed, mounted on microscope slides and were viewed and photographed at the indicated magnification on a Zeiss Axiovert 25 fluorescence microscope.

6. *DNA laddering assay*- Cells were treated with either TNF-α, sphingomyelinase or C_2-ceramide for 1 hour at concentrations identical to those used in the SREBP-1 maturation studies. The cells were then washed twice with minimal essential medium and refed with media A for 6 hours. The cells were harvested and genomic DNA was prepared as described (22). Genomic DNA was electrophoresed and stained with ethidium bromide.

7. *Electrophoretic Mobility Shift Assays*- Gel mobility shift assays were performed as follows. Each 20 µl reaction mixture contained 8-10 µg of nuclear protein plus a α-[^{32}P]–labeled 25-base pair oligonucleotide probe containing the SREBP-binding site (14) in binding buffer (10 mM Hepes, pH 7.5, 0.5 mM spermidine, 0.15 mM EDTA, 10 mM dithiothreitol, 0.35 mM sucrose). The reaction mixture was incubated at room temperature for 15 min and loaded directly onto a 6.5% polyacrylamide (49:0.6 acrylamide/bisacrylamide) gel in a buffer of 25 mM Tris borate (pH 8.0), 0.25 mM EDTA. In some experiments, antisera specific for SREBP or preimmune sera were added to reaction mixtures to determine the composition of protein-probe complexes. For these "supershift" assays, extracts were incubated with 1 µl of preimmune sera or an equal volume of anti-SREBP antisera at 4°C for 30 min prior to addition of α-[^{32}P]–labeled probe. In all experiments, proteins were separated by electrophoresis at 200 V for 2 h at room temperature. Gels were dried and exposed to Kodak XAR film with intensifying screens. Assays were repeated with nuclear

extracts obtained from three unique experiments and evaluated by phosphoimage analysis to ensure reproducibility of results.

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The invention has been described with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

What is claimed is:

1. An anti-lipemic drug comprising a therapeutically effective amount of at least one effect of the sterol regulatory element binding protein-1 (SREBP-1).

2. The anti-lipemic drug of claim 1, wherein the therapeutically effective amount is sufficient to reduce serum cholesterol level in a mammal compared to a suitable control mammal.

3. The anti-lipemic drug of claim 1, wherein the drug further comprises at least one synthetic or semi-synthetic inhibitor of an enzyme associated with cholesterol biosynthesis.

4. The anti-lipemic drug of claim 3, wherein the enzyme is HMG CoA reductase or HMG-CoA-synthetase.

5. The anti-lipemic drug of claim 1, wherein the drug further comprises at least one caspase.

6. The anti-lipemic drug of claim 5, wherein the caspase is cpp32 protease (caspase-3).

7. The anti-lipemic drug of claim 3, wherein the inhibitor is a drug selected from the group consisting of fluvastatin, simvastatin, lovastatin, pravastatin, mevinolin (compactin) atorvastatin or a derivative thereof.

8. The anti-lipemic drug of claim 2, wherein at least one of the SREBP-1 effectors is a sphingolipid, neutral sphingomyelinase (N-SMase) or a therapeutically effective fragment thereof.

9. The anti-lipemic drug of claim 8, wherein the sphingolipid is a ceramide.

10 The anti-lipemic drug of claim 9, wherein the ceramide is naturally occurring ceramide or any one of C-2, 4, 6, or 8 ceramide.

11. The anti-lipemic drug of claim 1, wherein the drug comprises a sphingolipid associated with an inhibitor of HMG CoA reductase or HMG-CoA-synthetase.

12. The anti-lipemic drug of claim 11, wherein the drug comprises the sphingolipid covalently linked to one of fluvastatin, simvastatin, lovastatin, pravastatin mevinolin (compactin) atorvastatin; or a derivative thereof.

13. The anti-lipemic drug of claim 12, wherein the sphingolipid is ceramide and the ceramide is linked to one of the inhibitors through a hydroxyl (-OH) group on the inhibitor.

14. The anti-lipemic drug of claim 13, wherein the hydroxyl (-OH) group on the inhibitor is covalently linked to the C-3 group of the ceramide.

15. The anti-lipemic drug of claim 14, wherein the anti-lipemic drug comprises covalently linked in sequence: 1) ceramide, 2) a heterobifunctional spacer group linked to the C-3 group of the ceramide, and 3) the hydroxyl (-OH) group of the fluvastatin, simvastatin, lovastatin, pravastatin, mevinolin (compactin), artorvastatin; or decivahi thereof linked to a reactive carbon atom on the heterobifunctional spacer.

16. The anti-lipemic drug of claim 1, wherein the drug comprises neutral sphingomyelinase (N-SMase) or a therapeutically effective fragment thereof; the N-

SMase or fragment being associated with an inhibitor of HMG CoA reductase or HMG CoA synthetase.

17. The anti-lipemic drug of claim 16, wherein the drug further comprises the neutral sphingomyelinase (N-SMase) or the fragment thereof covalently linked to one of fluvastatin, simvastatin, lovastatin, pravastatin, mevinolin (compactin) atorvastatin; or a derivative thereof.

18. The anti-lipemic drug of claim 1, wherein the drug comprises ceramide, neutral sphingomyelinase (N-SMase) or a therapeutically effective fragment thereof.

19. The anti-lipemic drug of claim 16 or 18, wherein the neutral sphingomyelinase (N-SMase) is encoded by a sequence having at least 70% sequence identity to the sequence represented by SEQ ID NO:1 or the completement thereof.

20. The anti-lipemic drug of claim 19, wherein the effective fragment of the neutral sphingomyelinase (N-SMase) comprises a sequence having at least 70% sequence identity to nucleotides 862 to 1414 of SEQ ID NO:1 or the completement thereof.

21. The anti-lipemic drug of claim 20, wherein the therapeutically effective fragment of the neutral sphingomyelinase (N-SMase) consists of nucleotides 862 to 1414 of SEQ ID NO:1 or the complement thereof.

22. The anti-lipemic drug of any one of claims 8-21, wherein the neutral sphingomyelinase (N-SMase) or the fragment thereof is linked to one of the inhibitors through an amide bond of the N-SMase or the fragment.

23. The anti-lipemic drug of any one of claims 8-22, wherein the anti-lipemic drug comprises covalently linked in sequence: 1) the neutral sphingomyelinase (N-SMase) or the therapeutically effective fragment thereof, 2) a heterobifunctional spacer linked to the amide group of the N-Smase or the fragment, and 3) the hydroxyl (-OH) group of the fluvastatin, simvastatin, lovastatin, pravastatin, mevinolin (compactin), artorvastatin or derivative thereof linked to a reactive carbon atom of heterobifunctional spacer.

24. The anti-lipemic drug of any one of claims 1-23, wherein the drug is specifically formulated for topical or related use.

25. The anti-lipemic drug of claim 24, wherein the drug further comprises components sufficient to provide the drug as a liposome formulation.

26. A method for modulating serum cholesterol level in a mammal, wherein the method comprises administering to the mammal a therapeutically effective amount of the anti-lipemic drug of any one of claims 1 to 25.

27. A method for modulating SREBP-1 levels in a mammal, wherein the method comprises administering to the mammal a therapeutically effective amount of the anti-lipemic drug of any one of claims 1 to 25.

28. A method for modulating LDL receptor levels in a mammal, the method comprising administering to the mammal a therapeutically effective amount of the anti-lipemic drug of any one of claims 1 to 25.

29. The method of claim 28, wherein the mammal is a recognized animal model for atherosclerosis or related disease.

30. The method of claim 29, wherein the mammal is a Watanabe heritable hyperlipidemic rabbit or an apolipoprotein E negative mouse.

31. The method of any one of claims 26-28, wherein the mammal is a primate.

32. The method of claim 31, wherein the primate is a human patient who has been diagnosed as having, is suspected of having, or is susceptible to a cholesterol related disorder.

33. The method of claim 32, wherein the cholesterol related disorder is at least one of hyperlipoproteinemia including hypercholesterolemia, stroke, obesity, cardiac disease including atherosclerosis, cerebral atherosclerosis, cholesterol ester storage disorder, liver disease including organ transplantation failure and cirrhosis; diseases of the biliary system, and viral infection facilitating encephalitis.

34. The method of claim 32 or 33, wherein the susceptibility of the human patient is related to a genetic or environmental pre-disposition to the cholesterol related disorder.

35. A method for treating a disorder in a mammal associated with high serum cholesterol levels, the method comprising administering to the mammal a therapeutically effective amount of the anti-lipemic drug of any one of claims 1-25.

36. The method of claim 35, wherein the mammal is a primate.

37. The method of claim 36, wherein the primate is a human patient who has been diagnosed as having, is suspected of having, or is susceptible to a high serum cholesterol levels.

38. A method for modulating serum cholesterol level in a mammal, wherein the method comprises administering to the mammal a therapeutically effective amount of the anti-lipemic drug of claim 1, wherein the SREBP-1 effector is neutral sphingomyelinase (N-SMase) or a therapeutically effective fragment thereof; or a sphingolipid.

39. The method of claim 38, wherein the mammal is a recognized animal model for atherosclerosis or a related disease.

40. The method of claim 39, wherein the mammal is a Watanabe heritable hyperlipidemic rabbit or an apolipoprotein E negative mouse.

41. A method for modulating SREBP-1 levels in a mammal, wherein the method comprises administering to the mammal a therapeutically effective amount of the anti-lipemic drug of claim 1, wherein the SREBP-1 effector is neutral sphingomyelinase (N-SMase) or a therapeutically effective fragment thereof; or a sphingolipid.

42. A method for modulating LDL receptor levels in a mammal, the method comprising administering to the mammal a therapeutically effective amount of the anti-lipemic drug of claim 1, wherein the SREBP-1 effector is neutral sphingomyelinase (N-SMase) or a therapeutically effective fragment thereof; or a sphingolipid.

43. The method of any one of claims 38-42, wherein the neutral sphingomyelinase (N-SMase) is encoded by a sequence having at least 70% sequence identity to the sequence represented by SEQ ID NO:1 or completement thereof.

44. The method of any one of claims 38-42, wherein the effective fragment of the neutral sphingomyelinase (N-SMase) comprises a sequence having at least 70%

sequence identity to nucleotides 862 to 1414 of SEQ ID NO:1 or completement thereof.

45. The method of any one of claims 38-42, wherein the sphingolipid is ceramide.

46. The method of any one of claims 38-42, wherein the mammal is a primate.

47. The method of claim 46, wherein the primate is a human patient who has been diagnosed as having, is suspected of having, or is susceptible to a cholesterol related disorder.

48. The method of claim 47, wherein the cholesterol related disorder is at least one of hyperlipoproteinemia including hypercholesterolemia, stroke, obesity, cardiac disease including atherosclerosis , cholesterol ester storage disorder, liver disease including organ transplantation failure and cirrhosis; and diseases of the biliary system.

49. The method of claim 47 or 48, wherein the susceptibility of the human patient is a related to a genetic or environmental pre-disposition to the cholesterol related disorder.

50. The method of any one of claims 26-49, wherein the anti-lipemic drug is provided as a liposome formulation.

51. The method of claim 50, wherein the liposome formulation is specifically adapted for hepatic administration.

52. The method of claim 50 or 51, wherein the liposome formulation is administered to liver orally, intramuscularly, intraperitoneally, or via a stent or related implementation.

53. The method of any one of claims 26-52, wherein each of the methods reduces serum cholesterol levels in the mammal by at least 20% when compared to a suitable control mammal as determined by a standard serum cholesterol assay.

54. The anti-lipemic drug of any one of claims 1-25, wherein the drug exhibits an ID_{50} of between from about 20% to 90% as determined in a standard *in vitro* HMG CoA reductase assay.

55. The anti-lipemic drug of claim 54, wherein the drug is capable of reducing serum cholesterol in the mammal by at least about 20% when compared to a suitable control mammal as determined by a standard serum cholesterol binding assay.

56. A method for detecting an effector of the sterol regulatory element binding protein-1 (SREBP-1), the method comprising:

a) providing a population of cells capable of expressing SREBP-1,

b) contacting the cells with a candidate effector in an amount sufficient to induce maturation of the SREBP-1,

c) culturing the cells in medium; and

d) detecting maturation of the SREBP-1 as indicative of the effector of the SREBP-1.

57. The method of claim 56, wherein the effector of SREBP-1 is tumor necrosis factor (TNF-α), neutral sphingomyelinase (N-SMase) or an effective fragment thereof, sphinogmyelin, ceramide, cpp32, or cholesterol.

58. The method of claim 56 or 57 further comprising monitoring LDL receptor activity as being indicative of the effector of the SREBP-1.

59. A method for detecting an effector of LDL receptor biosynthesis, the method comprising:

a) providing a population of cells responsive to ceramide and capable of expressing SREBP-1,

b) contacting the cells with a candidate effector in an amount sufficient to induce maturation of the SREBP-1,

c) culturing the cells in medium; and

d) detecting biosynthesis of the LDL receptor as being indicative of the effector of the LDL receptor.

60. The method of claim 58 or 59, wherein the effector of the LDL receptor is tumor necrosis factor (TNF-α), neutral sphingomyelinase (N-SMase) or an effective fragment thereof; sphinogmyelin, ceramide, cpp32, or cholesterol.

61. A method for determining therapeutic capacity of an effector of SREBP-1 for treating a cholesterol related disease in a mammal, the method comprising:

a) providing a population of cells capable of expressing SREBP-1,

b) contacting the cells with a candidate compound in an amount sufficient to induce maturation of the SREBP-1,

c) culturing the cells in medium; and

d) detecting maturation of the SREBP-1 as indicative of the therapeutic capacity of the effector in treating the disease.

62. The method of claim 61, wherein the cells are further capable of responding to an increase or decrease in intracellular ceramide levels.

63. The method of claim 61 or 62 further comprising monitoring LDL receptor activity as being indicative of the therapeutic activity of the candidate compound.

64. The method of claim 63, wherein the candidate compound is a neutral sphingomyelinase (N-SMase) or an effective fragment thereof; sphinogmyelin, ceramide, cpp32, or cholesterol.

65. The method of any one of claims 57-64, wherein the neutral sphingomyelinase (N-SMase) is encoded by a sequence having at least 70% sequence identity to the sequence represented by SEQ ID NO:1 or completement thereof.

66. The method of any one of claims 43-51, wherein the effective fragment of the neutral sphingomyelinase (N-SMase) comprises a sequence having at least 70% sequence identity to nucleotides 862 to 1414 of SEQ ID NO:1 or completement thereof.

67. A method for determining therapeutic capacity of any one of the anti-lipemic drugs of claims 1-25 for treating a cholesterol related disease in a mammal, the method comprising:

a) providing a population of cells capable of expressing SREBP-1,

b) contacting the cells with the anti-lipemic drug in an amount sufficient to induce maturation of the SREBP-1,

c) culturing the cells in medium; and

d) detecting maturation of the SREBP-1 as indicative of the therapeutic capacity of the anti-lipemic drug in treating the disease.

68. The method of claim 67, wherein the cells are further capable of responding to an increase or decrease in intracellular ceramide levels.

69. The method of claim 67 or 68 further comprising monitoring LDL receptor activity as being indicative of the therapeutic activity of the anti-lipemic agent.

70. The method of claim 69, wherein the anti-lipemic drug is a neutral sphingomyelinase (N-SMase) or an effective fragment thereof; sphinogmyelin, ceramide, cpp32, or cholesterol.

71. The method of claim 70, wherein the neutral sphingomyelinase (N-SMase) is encoded by a sequence having at least 70% sequence identity to the sequence represented by SEQ ID NO:1 or completement thereof.

72. The method of any one of claims 56-57, wherein the effective fragment of the neutral sphingomyelinase (N-SMase) comprises a sequence having at least 70% sequence identity to nucleotides 862 to 1414 of SEQ ID NO:1 or completement thereof.

73. A method for determining therapeutic capacity of any one of the anti-lipemic drugs of claims 1-25 in a Watanabe heritable hyperlipidemic rabbit or apolipoprotein and negative mouse, the method comprising:

a) administering at least one of the anti-lipemic drugs to the rabbit or mouse in an

amount sufficient to reduce serum cholesterol levels by at least from about 10 to 20%

as determined by a standard cholesterol assay; and

b) detecting the serum cholesterol reduction in the rabbit or mouse as being indicative

of the therapeutic capacity of the anti-lipemic drug to treat the cholesterol related disease.

74. A method for modulation production of the amyloid precursor protein (ßAPP) comprising administering to a subject mammal a therapeutically effective amount of any one of the anti-lipemic drugs of claims 1-25.

75. A method for modulating fatty acid synthesis, the method comprising administering to a subject mammal a therapeutically effective amount of any one of the anti-lipemic drugs of claims 1-25.

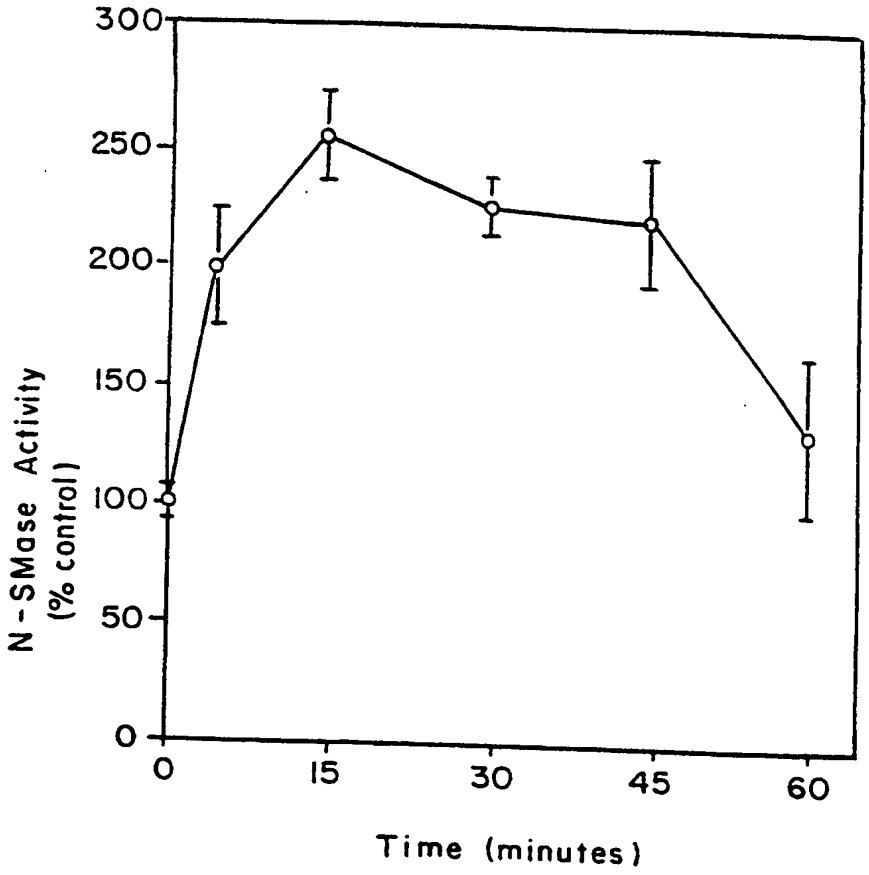


FIG. 1

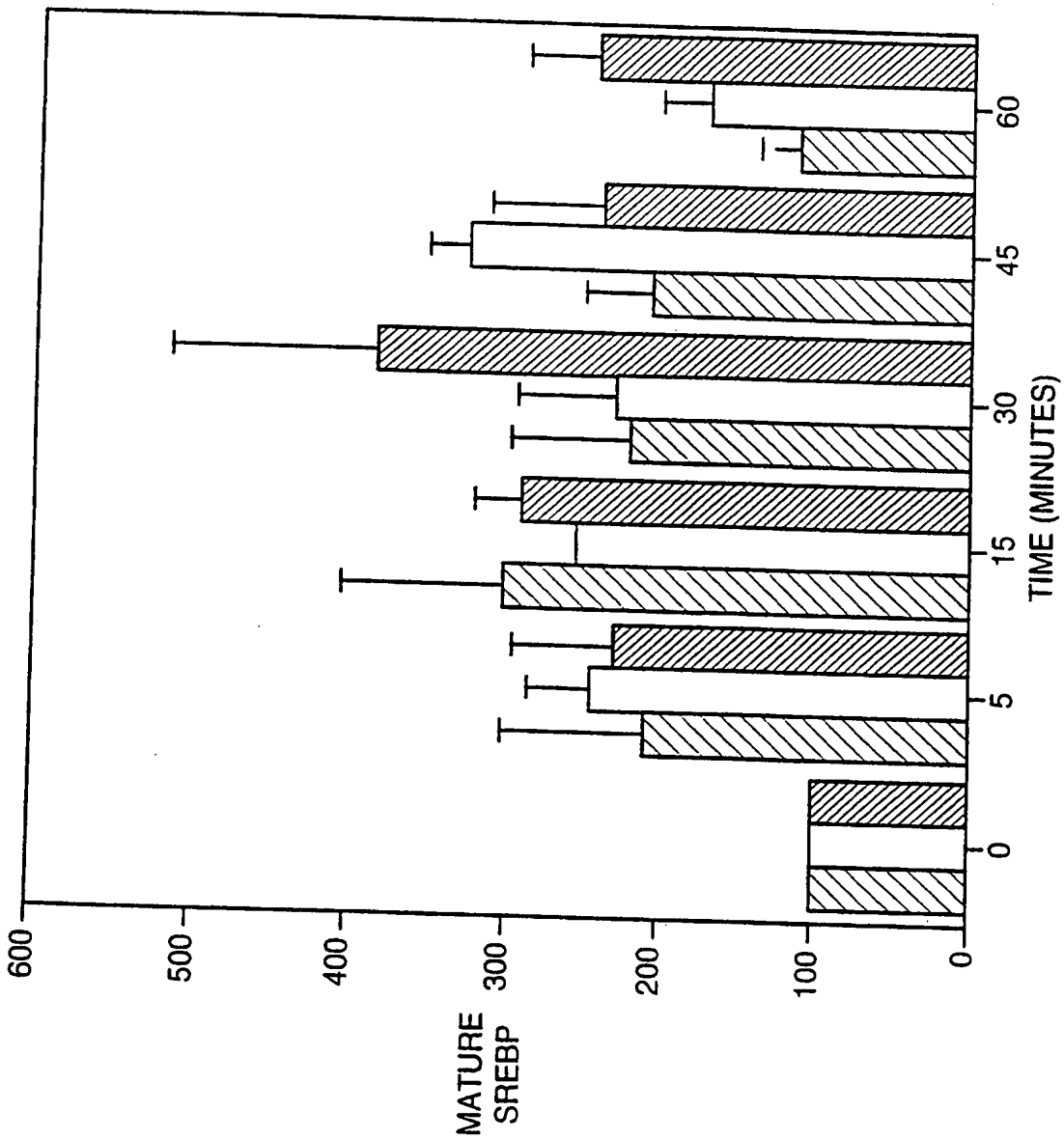

MATURE SREBP
600
500
400
300
200
100
0
0
5
15
30
45
60
TIME (MINUTES)

FIG. 2A

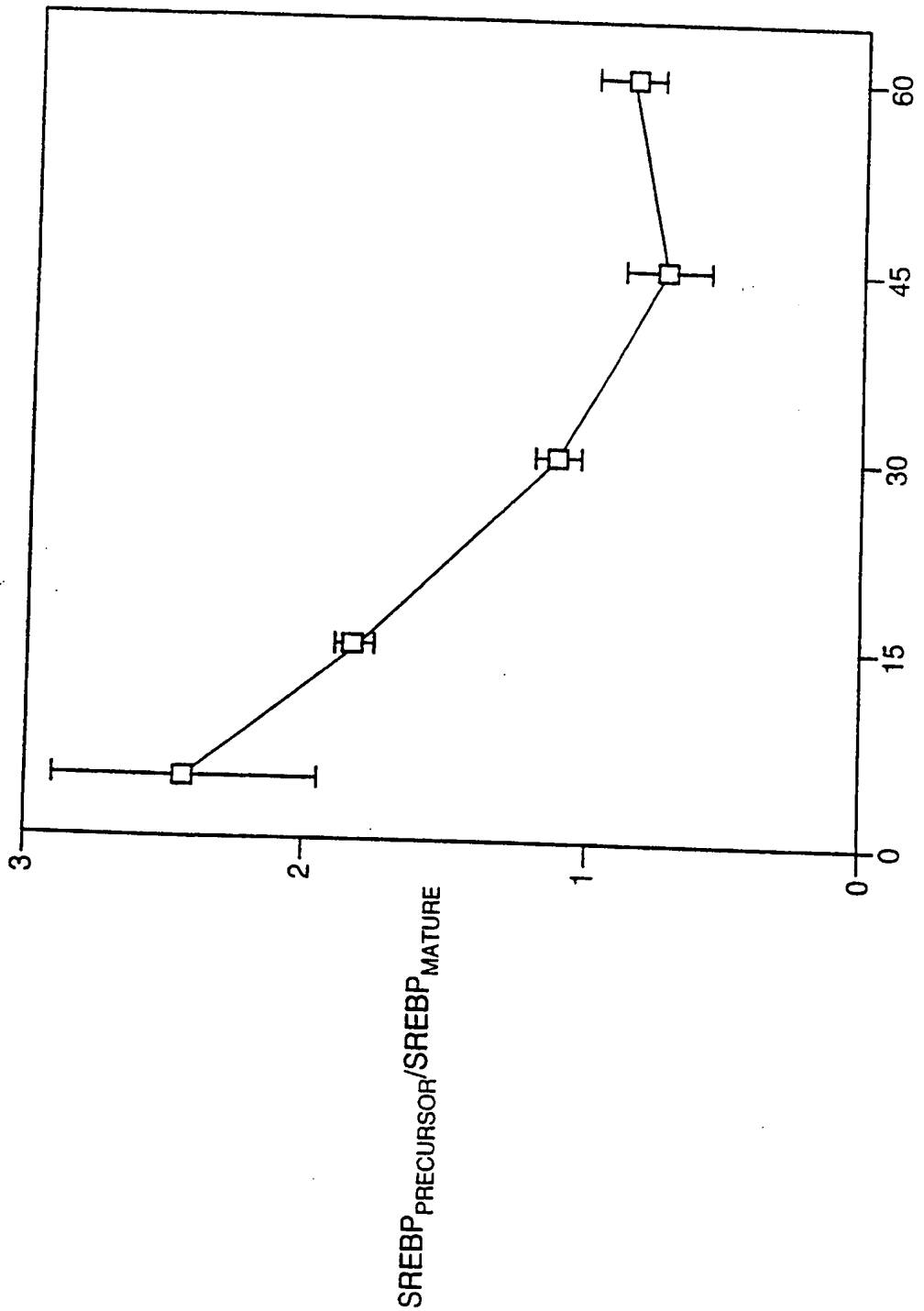
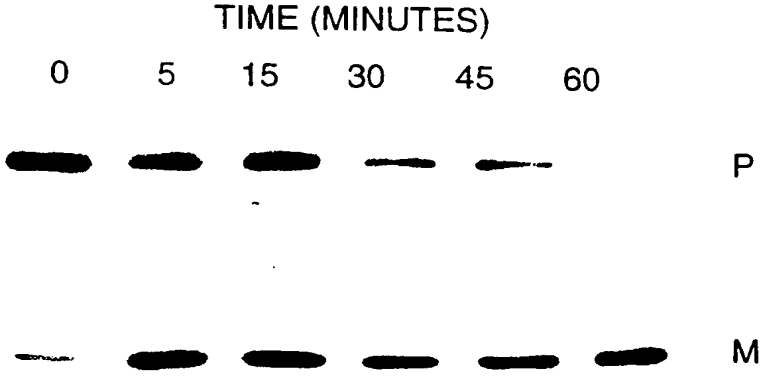
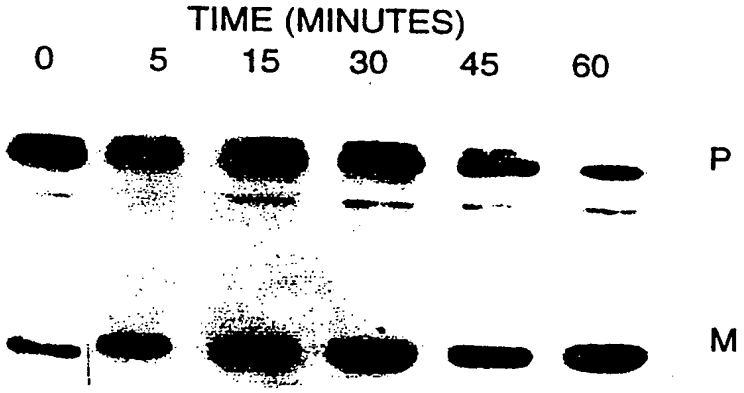


FIG. 2B



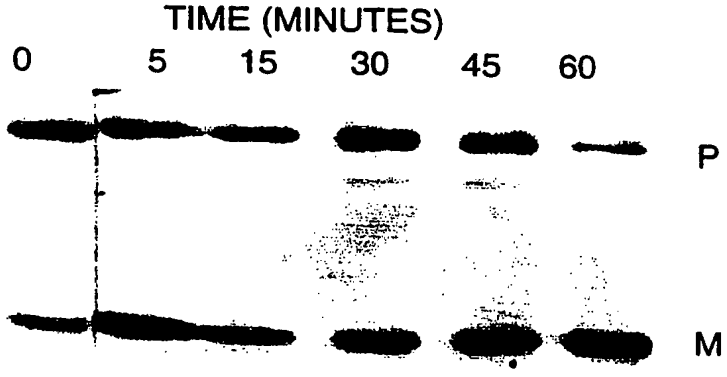
TNF-α

FIG. 2C-1



SPHINGOMYELINASE

FIG. 2C-2



C_2-CERAMIDE

FIG. 2C-3

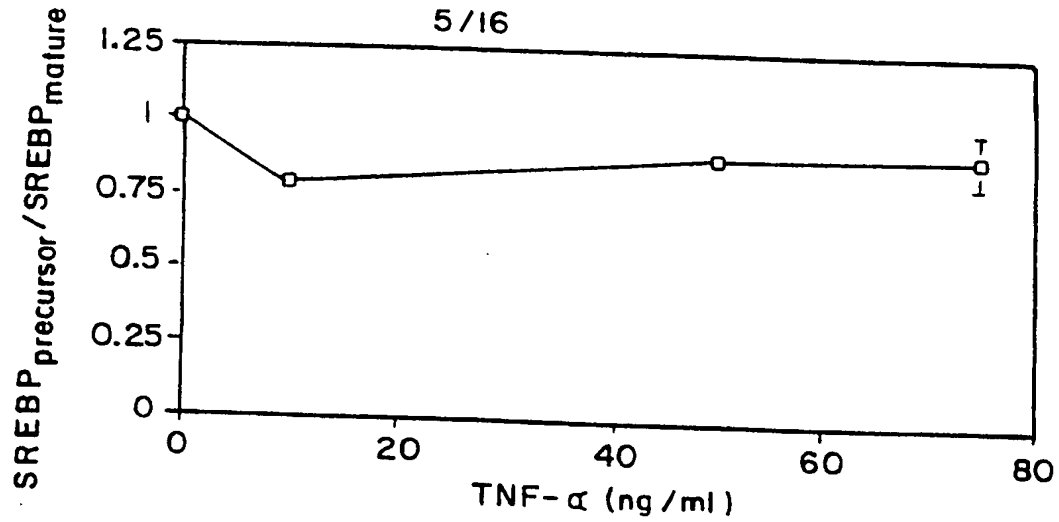


FIG. 3A

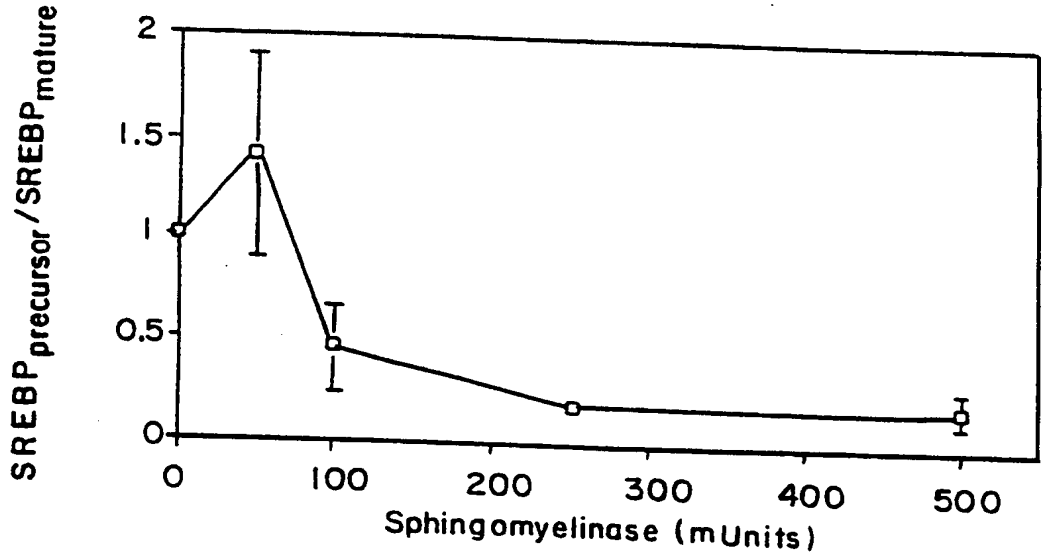


FIG. 3B

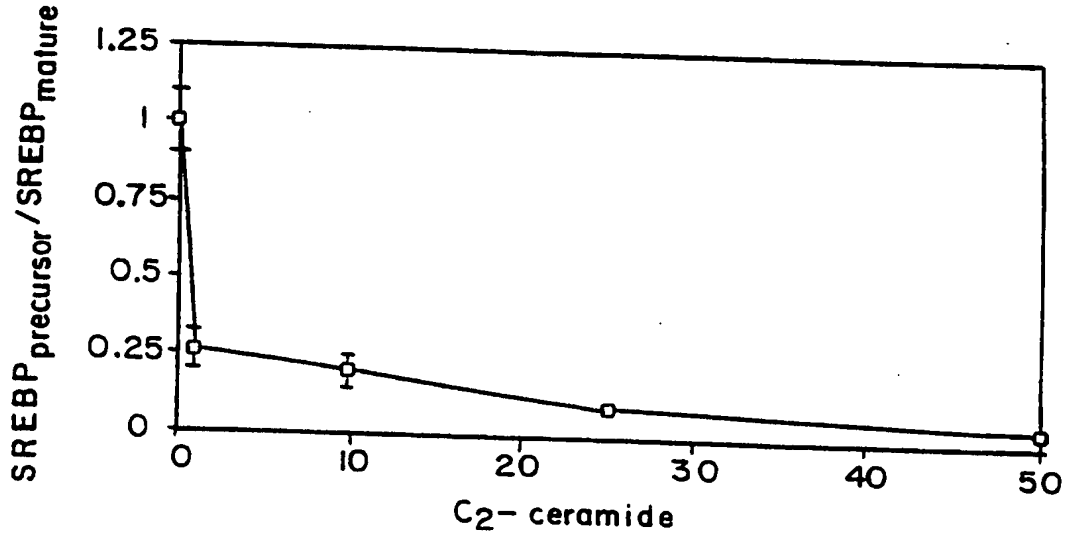


FIG. 3C

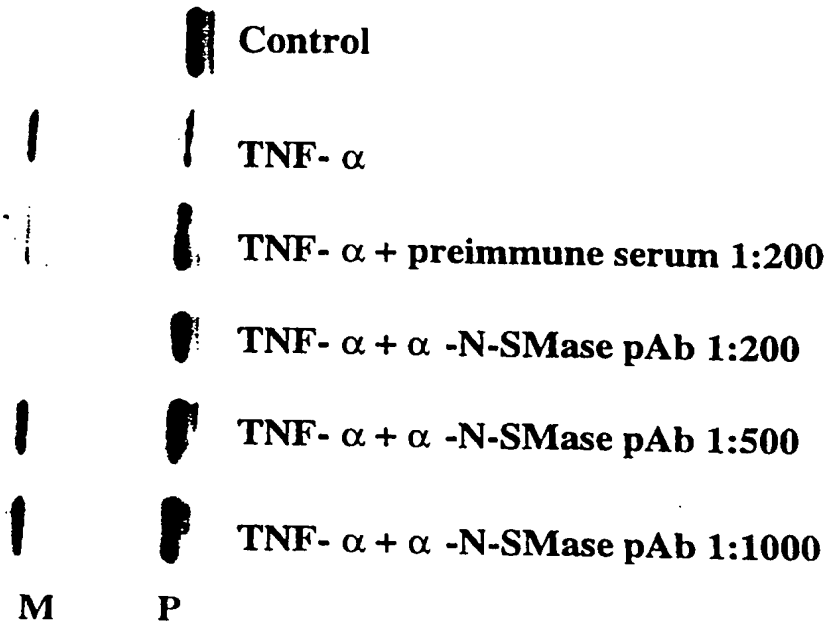


FIG. 4

FIG. 5A

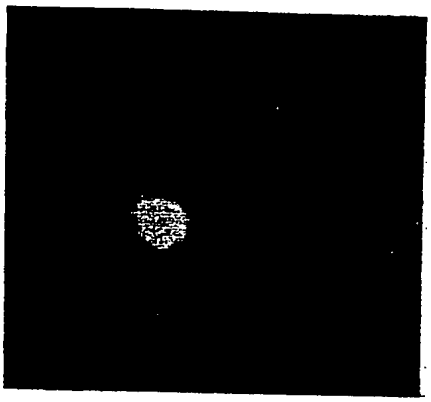


FIG. 5B

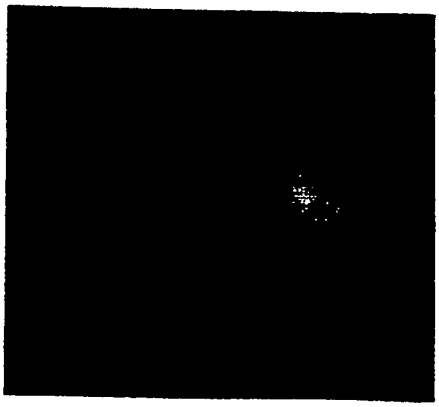


FIG. 5C

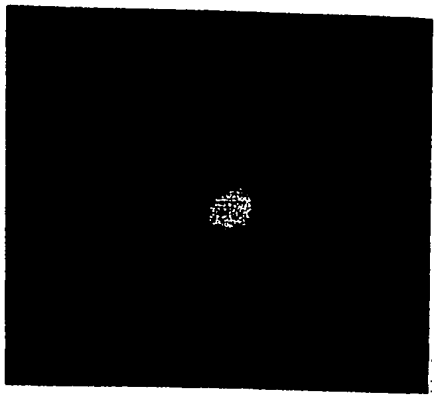


FIG. 5D

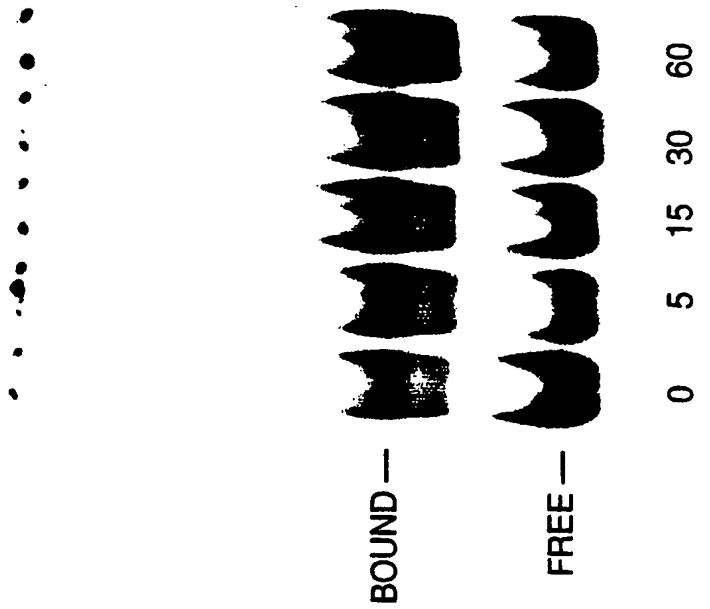


FIG. 6A

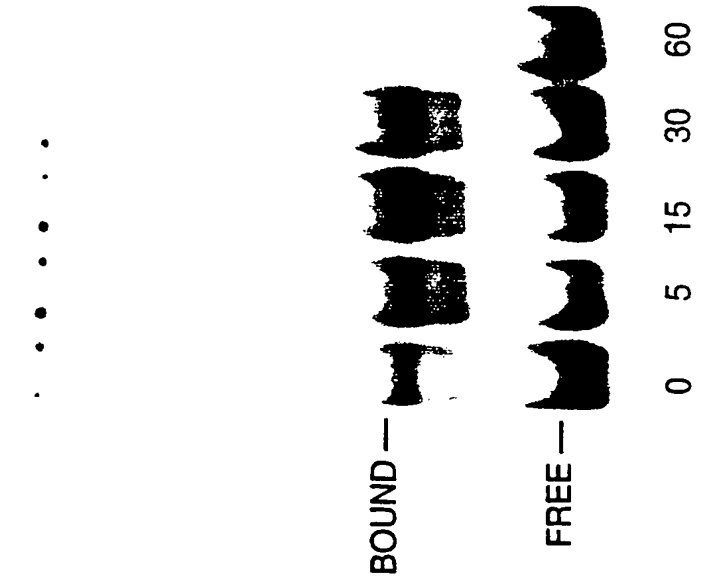


FIG. 6B

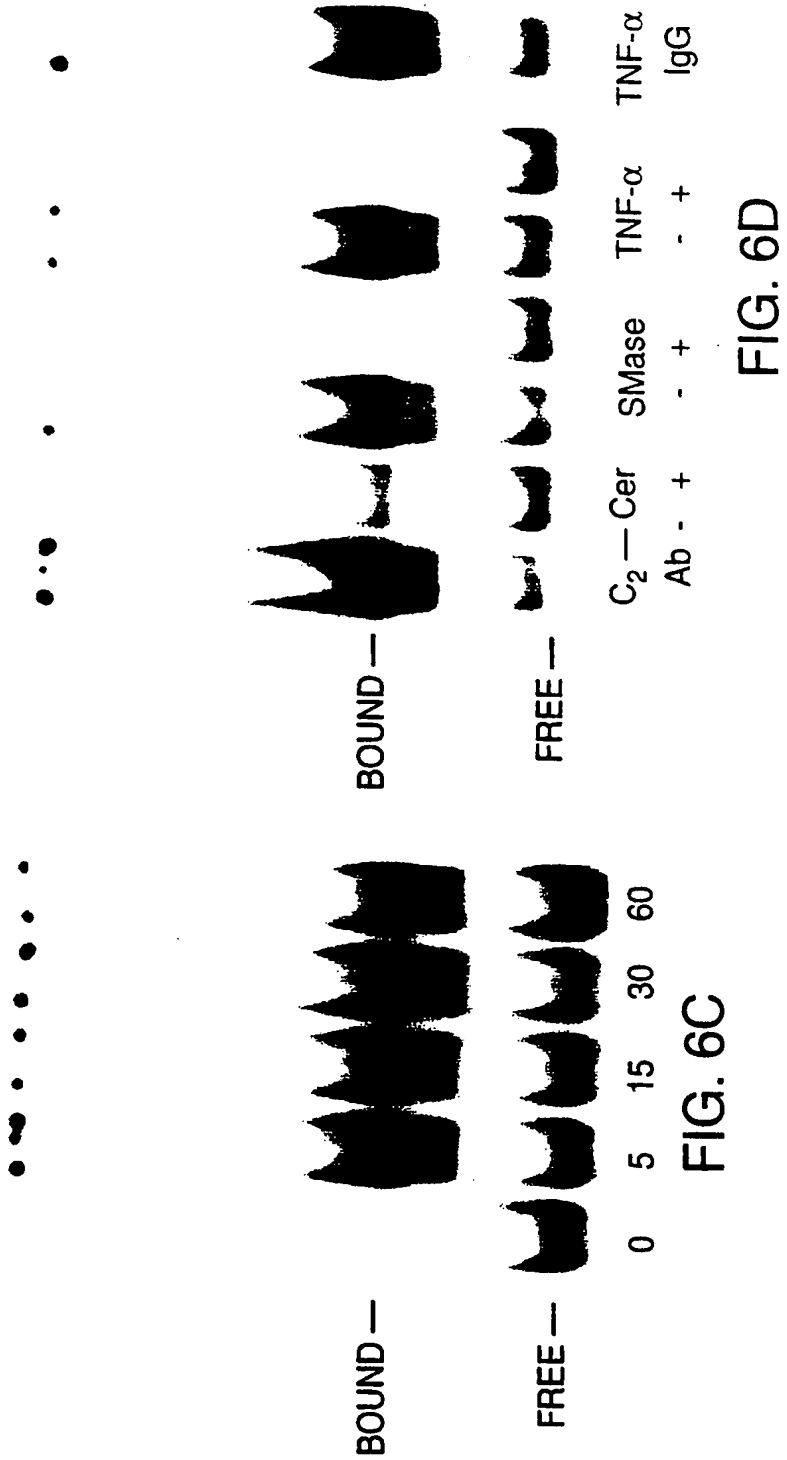


FIG. 6C

FIG. 6D

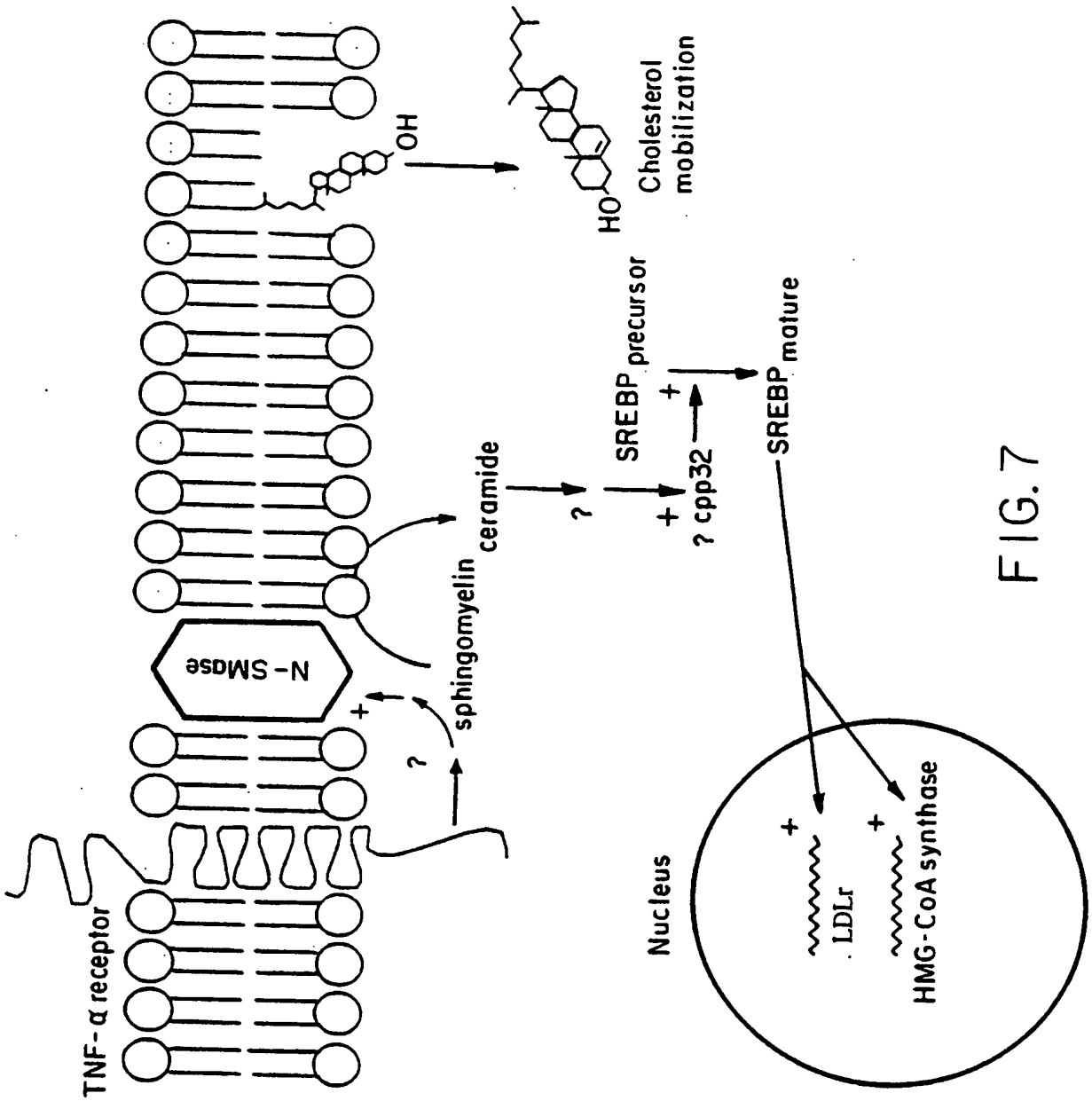


FIG. 7

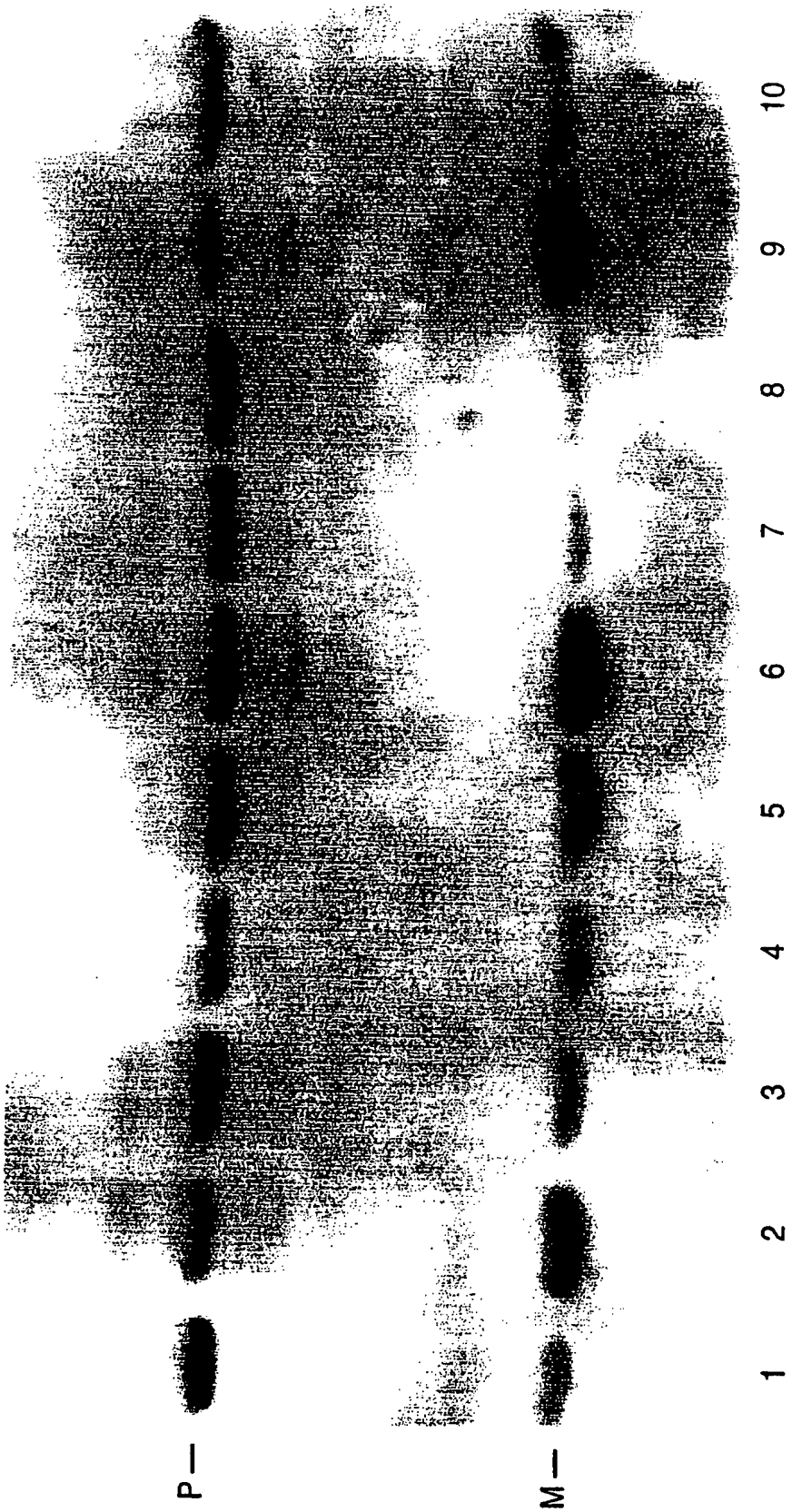


FIG. 8

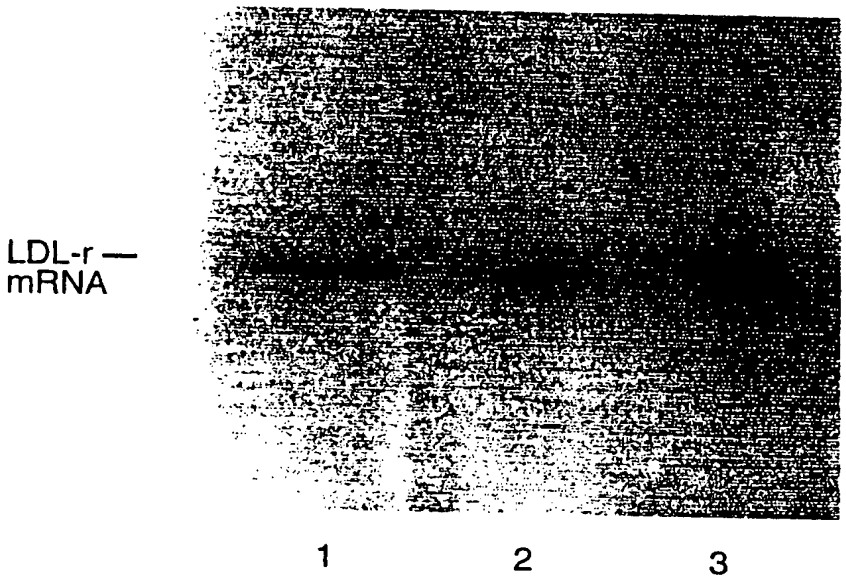


FIG. 9

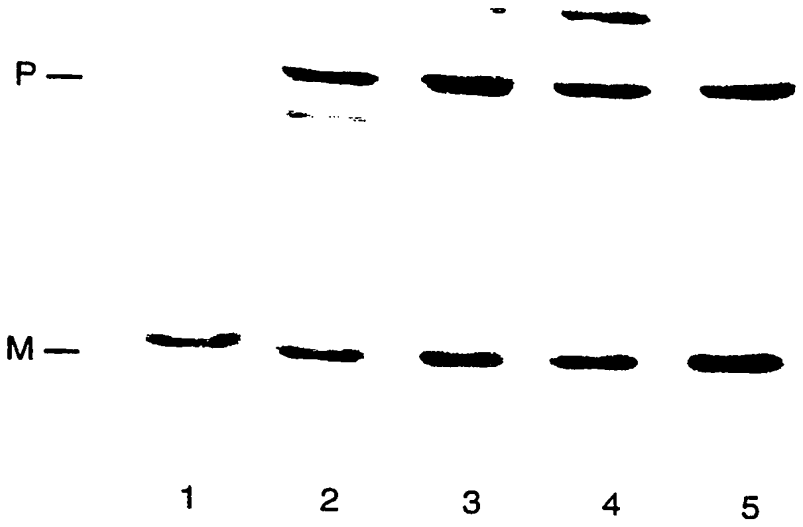


FIG. 10

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ATGATGACAT ATCACGAAAC GCGCGCGTTG GCTCAAAGCG ACTTACAGCA ACTCTATGCG    60
GCACTTGAAA CAACTGAATT TGGCGCTTAC TTTGCGACAC CCGCTGATGA TACTTTACGT   120
TTTGGCATTG GCGCAATCGC TACGGCAAAA ACGGCTCAGG CATTACAAGG TGCGGTTGTT   180
TTTGGTGCGC AGTCATTTGA TGAACAAGAG TACCCGCAGT CTGAATTGAT GGCGGGTTTT   240
TGGTTTTGTCC CCGAAGTGAT GGTGACCATC GCGGCAGATA AAATCACGTT CGGATCAGAT   300
ACCGTATCTG ATTTTACGAC GTGGCTGGCG CAGTTCGTGC CAAACAGCCC AAATACGGTG   360
ACCACTAGTC ATGTGACAGA TGAAGTGGAT TGGATCGAAC GGACAGAGAA TTTGATTGAT   420
ACCTTAGCCA TCGATCAAAC CTTAGCCAAA GTCGTTTTTG GTCGGCAACA GACCCTGCAG   480
TTATCCGACA CGTTACGACT GGCACAAATT ATTCGTGCGT TAGCTGAGCA GGCGAATACG   540
TATCATGTGG TTTTAAAGCG ACATGATGAA TTGTTTATTT CAGCAACACC GGAACGGTTA   600
GTGGCTATGT CAGGTGGTCA GATCGCTACG GCGGCGGTCG CTGGGACAAG CCGGCGCGGG   660
ACGGATGGCG CTGACGATAT CGCGTTAGGC GAAGCGTTGT TAGCCAGTCA GAAAAACCGC   720
ATTGAACATC AATATGTCGT GGCAAGTATC ACGACACGCT TGCAAGACGT GACGACGTCG   780
CTAAAGGTGC CGGCCATGCC AAGTTTACTC AAAAATAAGC AAGTTCAGCA TTTGTACACA   840
CCAATTACAG GGGACATTGC GGCACATTTA AGTGTGACCG CGATTGTTGA CCGCTTGCAT   900
CCAACACCAG CACTGGGTGG CGTCCCACGT GAAGCGGCCC TGTATTACAT TGCGACCCAT   960
GAGAAGACAC CTCGTGGCTT GTTTGCAGGT CCTATTGGCT ATTTTACCGC AGATAATAGT  1020
GGGGAATTTG TGGTTGGCAT CCGTTCCATG TATGTGAATC AAACGCAGCG ACGAGCAACT  1080
TTATTTGCTG GTGCCGGGAT TGTGGCTGAC TCCGATGCGC AACAAGAATA TGAAGAAACT  1140
GGGTTGAAAT TTGAACCCAT GCGGCAATTG TTAAAGGACT ACAATCATGT CGAATGA     1197
```

FIG. 11

```
Met Met Thr Tyr His Glu Thr Arg Ala Leu Ala Gln Ser Asp Leu Gln
 1               5                  10                  15
Gln Leu Tyr Ala Ala Leu Glu Thr Thr Glu Phe Gly Ala Tyr Phe Ala
            20                  25                  30
Thr Pro Ala Asp Asp Thr Leu Arg Phe Gly Ile Gly Ala Ile Ala Thr
        35                  40                  45
Ala Lys Thr Ala Gln Ala Leu Gln Gly Ala Val Phe Gly Ala Gln Ser
    50                  55                  60
Phe Asp Glu Gln Glu Tyr Pro Gln Ser Glu Leu Met Ala Gly Phe Trp
65                  70                  75                  80
Phe Val Pro Glu Val Met Val Thr Ile Ala Ala Asp Lys Ile Thr Phe
                85                  90                  95
Gly Ser Asp Thr Val Ser Asp Phe Thr Thr Trp Leu Ala Gln Phe Val
            100                 105                 110
Pro Lys Gln Pro Asn Thr Val Thr Thr Ser His Val Thr Asp Glu Val
        115                 120                 125
Asp Trp Ile Glu Arg Thr Glu Asn Leu Ile Asp Thr Leu Ala Ile Asp
    130                 135                 140
Gln Thr Leu Ala Lys Val Val Phe Gly Arg Gln Gln Thr Leu Gln Leu
145                 150                 155                 160
Ser Asp Thr Leu Arg Leu Ala Gln Ile Ile Arg Ala Leu Ala Glu Gln
                165                 170                 175
Ala Asn Thr Tyr His Val Val Leu Lys Arg His Asp Glu Leu Phe Ile
            180                 185                 190
Ser Ala Thr Pro Glu Arg Leu Val Ala Met Ser Gly Gly Gln Ile Ala
        195                 200                 205
Thr Ala Ala Val Ala Gly Thr Ser Arg Arg Gly Thr Asp Gly Ala Asp
    210                 215                 220
Asp Ile Ala Leu Gly Glu Ala Leu Leu Ala Ser Gln Lys Asn Arg Ile
225                 230                 235                 240
Glu His Gln Tyr Val Val Ala Ser Ile Thr Thr Arg Leu Gln Asp Val
                245                 250                 255
Thr Thr Ser Leu Lys Val Pro Ala Met Pro Ser Leu Leu Lys Asn Lys
            260                 265                 270
Gln Val Gln His Leu Tyr Thr Pro Ile Thr Gly Asp Ile Ala Ala His
        275                 280                 285
Leu Ser Val Thr Ala Ile Val Asp Arg Leu His Pro Thr Pro Ala Leu
    290                 295                 300
Gly Gly Val Pro Arg Glu Ala Ala Leu Tyr Tyr Ile Ala Thr His Glu
305                 310                 315                 320
Lys Thr Pro Arg Gly Leu Phe Ala Gly Pro Ile Gly Tyr Phe Thr Ala
                325                 330                 335
Asp Asn Ser Gly Glu Phe Val Val Gly Ile Arg Ser Met Tyr Val Asn
            340                 345                 350
Gln Thr Gln Arg Arg Ala Thr Leu Phe Ala Gly Ala Gly Ile Val Ala
        355                 360                 365
Asp Ser Asp Ala Gln Gln Glu Tyr Glu Glu Thr Gly Leu Lys Phe Glu
    370                 375                 380
Pro Met Arg Gln Leu Leu Lys Asp Tyr Asn His Val Glu
385                 390                 395
```

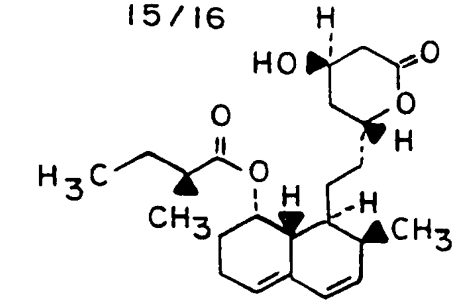
FIG. 12

COMPOUND	TARGET	RESULT
CERAMIDE (C-2, C-6, C-8, NATURAL) HEXOSE - G-P	LIVER	INCREASE LDL RECEPTORS (DECREASE PLASMA CHOLESTEROL)
CERAMIDE ~ HEXOSE - G-P	LIVER	INCREASE LDL RECEPTORS (DECREASE HMG-GA REDUCTASE)
N-SMase ~ LIPOSOME (TOPICAL USE ONLY)	SKIN	DECREASE PLANAR XANTHOMA (E.G., EYELIDS)
PHH1 ~ HEXOSE – 6 – P ± STATINS	LIVER	INCREASE LDL RECEPTORS (DECREASE PLASMA CHOLESTEROL)
PHH–II ~ HEXOSE – 6 – P ± STATINS	LIVER	IBID.
PHH–1 ~ D – PDMP	KIDNEY	KILL TUMORS (APOPTOSIS) AND REDUCE CHOLESTEROL SUPPLY
PHH–1 ~ D – PDMP	KIDNEY	KILL AND/OR PRESENT TUMORS AND REDUCE CHOLESTEROL SUPPLY

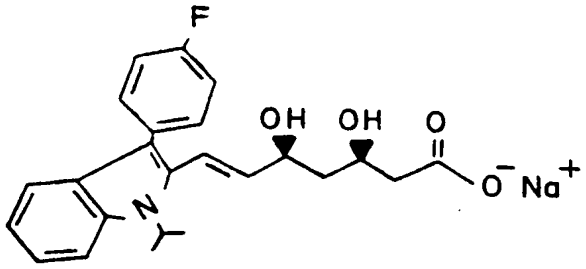
~ = CHEMICAL BOND

FIG. 13

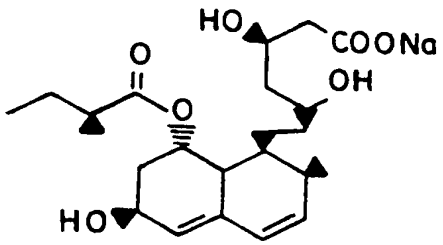
FIG. 14



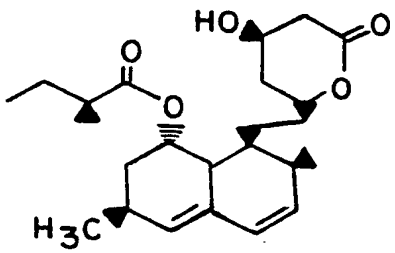
Mevastatin



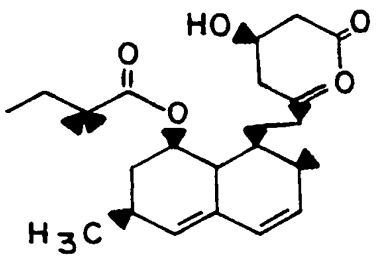
Fluvastatin



Pravastatin



Lovastatin



Simvastatin

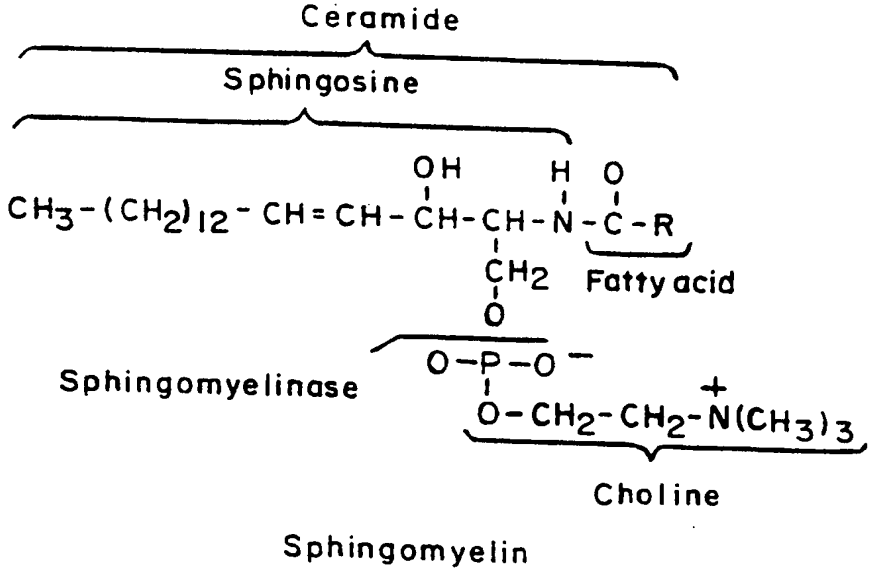
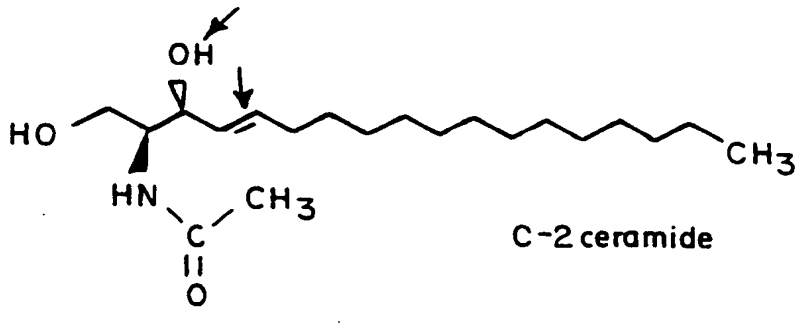


FIG. 15A



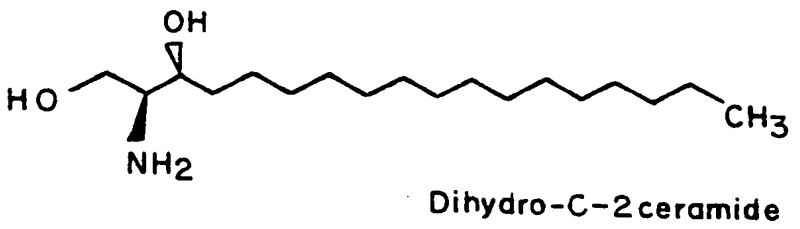


FIG. 15B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04657

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 9/00, 9/50; A01N , 43/04, 61/00; A61K 38/28
US CL : 435/183, 219; 514/1, 4, 27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/183, 219; 514/1, 4, 27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, CAPLUS, BIOSIS, MEDLINE, EMBASE, TOXLINE, TOXLIT, USPATFULL, IFIPAT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FRISHMAN et al. Lovastatin: An HMG-CoA reductase inhibitor for lowering cholesterol, Medical Clinics of North America, 1989, Vol. 73,NO.2, pages 437-448, see entire article.	1-21 and 25
Y	DUANE, W.C. et al. Simvastatin, a competitive inhibitor of HMG Coenzyme A reductase lowers cholesterol saturation index of gall bladder bile. Hepatology, 1988, Vol. 8, no.5, pages 1147-1150, especially 1148.	1-21 and 25
Y	MITCHELL, J.C. et al. Effects of lovastatin on biliary lipid secretion and bile acid metabolism in humans. J. Lipid Res., 1991, Vol. 32, pages 71-78 (see entire article).	1-21 and 25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
25 MAY 2000

Date of mailing of the international search report
15 JUN 2000

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04657

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 3,876,789 (SANTILLI ET AL.) 08 April 1975 (04/08/75), see entire document.	1-21 and 25
Y	US 5,498,696 (BRIGGS ET AL.) 12 March 1996 (03/12/96), see entire document.	1-21 and 25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/04657

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 22-24, 26-28, 31, 35, 43-46, 50, 53, 54, 65-67, 69, 72-75
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.